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EDITORIAL ANNOUNCEMENT

THE Editors are happy to announce that Professor R. E. Zirkle of the Committee on Biophysics, University of Chicago, has agreed to join them as a co-editor. It is expected that Volume 11, which is now in active preparation, will appear under their joint Editorship at the end of 1960. The general policy and aims of the series will remain the same as stated in the Prefaces of previous volumes.

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1

THE KINETICS OF REACTIONS BETWEEN HAEMOGLOBIN AND GASES

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1

THE KINETICS OF REACTIONS BETWEEN HAEMOGLOBIN AND GASES

Q. H. Gibson

I. Introduction

Work in this field has always required special methods, and it is perhaps the experimental difficulties that have tended to confine studies on the reactions of haemoglobin with gases to a few laboratories. The scope of this article has been determined partly by the review of George (1956) which has dealt, as would be expected from its author's position in the field, particularly well with the reactions of methaemoglobin with ligands. The form adopted here is primarily descriptive, since the work of measurement is not yet sufficiently far advanced to allow adequate theoretical treatment. A brief introduction recapitulating some of the more prominent facts about haemoglobin is followed first by a section on methods and then by an account of the chief reactions of haemoglobin with gases which have been studied so far. A section at the end is devoted to pointing out some inconsistencies and anomalies in the existing observations.

II. General Properties of Haemoglobin and Intermediate Compound Theory

Mammalian haemoglobins are proteins with a molecular weight of about 65,000, containing 0·33 per cent Fe, and hence 4 iron atoms per molecule. The average molecular size was first satisfactorily established by Adair (1924) who also showed, using osmotic pressure measurements, that oxyhaemoglobin and reduced haemoglobin have the same molecular weight. His estimate was later confirmed by Svedberg and Fåhraeus (1926) whose ultracentrifugal measurements showed, in addition, that the solutions were substantially homogeneous, each molecule containing 4 iron atoms. The sigmoid shape of the equilibrium curve (Fig. 1) relating percentage saturation of haemoglobin to partial pressure of oxygen or carbon monoxide has been known for more than 50 years, and its value in assisting efficient oxygen transport in the body has been appreciated since the work of Barcroft described in the classic *Respiratory Function of the Blood* (1st Ed., 1914).

There have been many attempts to account for the form of the equilibrium curve, most of which are now of historic interest only.

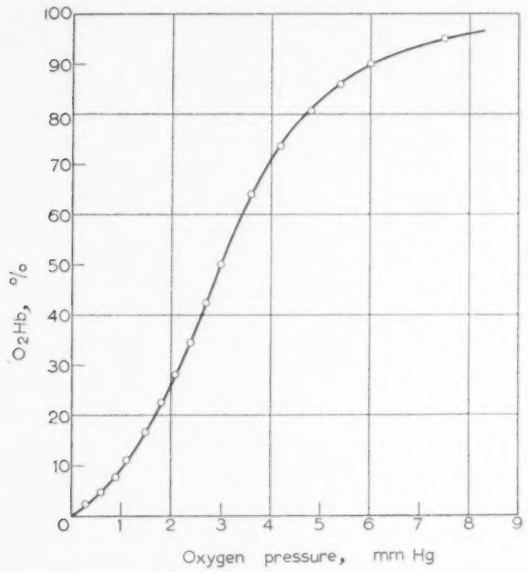

Fig. 1. Oxygen dissociation curve of sheep haemoglobin *p*H 9·3, 18°C. Ordinate, percentage O_2Hb; abscissa, oxygen pressure mm. Hg. Data of FORBES and ROUGHTON (from ROUGHTON, F. J. W., 1949).

The basis generally accepted at present is due to ADAIR (1925) who suggested that the combination of ligands with haemoglobin takes place in four separate steps:

$$Hb_4 + X \underset{x_1}{\overset{x_1'}{\rightleftharpoons}} Hb_4X \qquad \frac{x_1'}{x_1} = X_1 \qquad \ldots.(1)$$

$$Hb_4X + X \underset{x_2}{\overset{x_2'}{\rightleftharpoons}} Hb_4X_2 \qquad \frac{x_2'}{x_2} = X_2 \qquad \ldots.(2)$$

$$Hb_4X_2 + X \underset{x_3}{\overset{x_3'}{\rightleftharpoons}} Hb_4X_3 \qquad \frac{x_3'}{x_3} = X_3 \qquad \ldots.(3)$$

$$Hb_4X_3 + X \underset{x_4}{\overset{x_4'}{\rightleftharpoons}} Hb_4X_4 \qquad \frac{x_4'}{x_4} = X_4 \qquad \ldots.(4)$$

where X represents the ligand combining with haemoglobin, x_1', x_2', x_3' and x_4' are the velocity constants for the combination of the first, second, third and fourth molecules of the ligand respectively, x_1, x_2, x_3 and x_4 the dissociation velocity constants for the dissociation of the fourth, third, second and first molecules of the ligand leaving the complex and X_1, X_2, X_3 and X_4 are the equilibrium constants for the intermediate reactions.

It has become conventional to use certain letters in writing reactions involving particular ligands: thus k', k and K are used for reactions with oxygen, y represents oxyhaemoglobin concentration, and p partial pressure of oxygen. For carbon monoxide l', l, L, z and q are used, and for nitric oxide GIBSON AND ROUGHTON (1957a) have recently suggested j', j and J. The relation between certain of the constants is also important: the relative affinity for CO and O_2 when no free gas-binding groups are present is given by $L_4/K_4 = M$, and for NO and CO, $J_4/L_4 = N$. Because of the gasometric methods used in equilibrium work, the equilibrium constants are often expressed in units of gas partial pressures. The kinetic constants are commonly given in the usual units of sec^{-1} and l/mole sec for first and second-order constants respectively.

Equations (1) and (2) can be used to fit the sigmoid equilibrium curves obtained experimentally on the assumption that the oxygenation of one of the haems facilitates the oxygenation of the others—that is there is haem–haem interaction. Although it was clear in 1925 that such haem–haem interaction must be assumed it was not until 1955 that OTIS, ROUGHTON, and LYSTER succeeded in obtaining observations of sufficient range and precision to evaluate the four equilibrium constants and hence the extent of haem–haem interaction at each stage. Working with sheep haemoglobin at pH 9·1 they found that there was little interaction until 3 gas molecules had combined, but that thereafter a great increase in affinity occurred, the fourth molecule being some 250 times more firmly bound than the first. LYSTER (Ph.D. Thesis, Cambridge) has recently examined horse and human haemoglobins and has found that in these species interaction seems to occur between pairs of haems, the first and third molecules of oxygen being loosely bound, the second and fourth tightly held.

These successes in applying the intermediate compound hypothesis to the treatment of equilibrium data naturally stimulated interest in its application to the kinetics of the reactions, which until 1948 had been treated with fair success by assuming the processes involved to be of the form

$$\mathrm{Hb} + \mathrm{O_2} \underset{k}{\overset{k'}{\rightleftharpoons}} \mathrm{HbO_2} \qquad \ldots.(5)$$

giving rise to the differential equation

$$\mathrm{d}y/\mathrm{d}t = k'(\alpha - y)(\beta - y) - ky \qquad \ldots.(6)$$

where α = total oxygen in combination and in solution

β = total gas combining capacity of haemoglobin

A graphical method of solution of this equation for the case of oxygen combining with reduced haemoglobin has been given by HARTRIDGE

and ROUGHTON (1925); in the important case where the oxygen concentration is held at zero in the presence of dithionite it reduces to the ordinary first order equation

$$dy/dt = -ky \qquad \ldots.(7)$$

The general set of differential equations corresponding to equations (1)–(4) can be most compactly written down by introducing the extra symbols at time t, $[Hb_4] = r$, $[Hb_4X] = s$, $[Hb_4X_2] = u$, $[Hb_4X_3] = v$, $[Hb_4X_4] = w$, and $[Hb]_4$ (at $t = 0$) $= r_0$. Where the ligand is oxygen:

$$dr/dt = k_1 s - k_1' rp \qquad \ldots.(8)$$

$$ds/dt = (k_1' r - k_2' s)p + k_2 u - k_1 s \qquad \ldots.(9)$$

$$du/dt = (k_2' s - k_3' u)p + k_3 v - k_2 u \qquad \ldots.(10)$$

$$dv/dt = (k_3' u - k_4' v)p + k_4 w - k_3 v \qquad \ldots.(11)$$

and

$$w = r_0 - (r + s + u + v) \qquad \ldots.(12)$$

The full set of equations is in general quite intractable except by numerical methods using electronic computing: particular methods of solution have, however, been given by GIBSON and ROUGHTON (1957b) for the case where the back reactions $k_1 s$, $k_2 u$, etc. are small enough to be neglected, while the case where the ligand concentration exceeds the haemoglobin concentration sufficiently so that it may be treated as constant throughout the reaction *and* the back reaction is negligible is analytically soluble (BATEMAN, 1910).

Although the mathematical difficulties in using these equations are very considerable, the greatest obstacle to their general application is that at least 4 constants are involved in the description of any individual time–concentration curve, and with so many constants a very large number of sets of numerical values can be found each of which is able to reproduce the experimental curves very closely. It is at present only possible to treat fully certain special cases where values of some of the constants can be obtained independently by special methods and so allow the fitting of equations (8)–(12) to start with not more than two unknowns. In the less favourable cases equation (6) is still useful as an approximation but must be regarded as descriptive of the observed overall reaction only and not necessarily giving even a rough estimate of the size of any individual constant.

Although it can no longer be regarded as having any physical basis HILL's equation is often useful in describing equilibrium data. This equation was put forward in 1910 (at a time when the molecular weight of haemoglobin had not been satisfactorily determined) and assumed the existence of haemoglobin molecules containing variable numbers of

haems. These haems were supposed to show infinite interaction, leading to the equilibrium equation

$$y = Kp^n/(1 + Kp^n) \qquad \ldots.(13)$$

where n is the average number of haems per molecule. The value of n in HILL's equation serves as a ready means of comparing the extent of interaction in different haemoglobin preparations. The corresponding kinetic equation analogous to (6) is

$$dy/dt = k'(\alpha - y)^n(\beta - y) - ky \qquad \ldots.(14)$$

This equation has not found any practical application as no example of a haemoglobin reaction with appropriate kinetic properties has so far been described.

III. METHODS

1. *Flow methods*

By far the most important single step towards the development of the methods which have been and are being used in the study of haemoglobin was taken by HARTRIDGE and ROUGHTON (1923a) when they

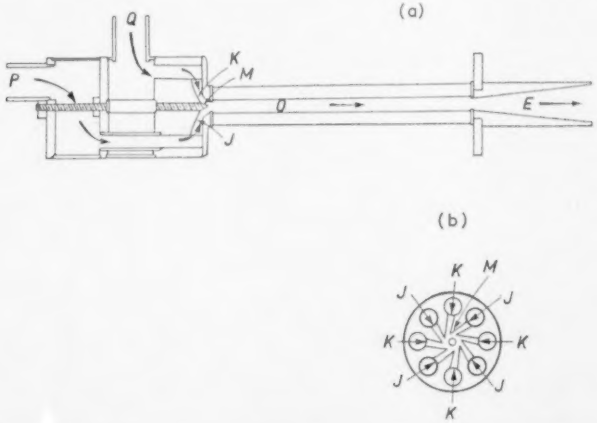

Fig. 2. (a). Diagrammatic longitudinal section of the mixing apparatus used by HARTRIDGE and ROUGHTON (1925). The reduced haemoglobin and the oxygenated water enter respectively through the pipes P and Q. They are then forced through the four jets J and the four jets K respectively, and enter the mixing chamber M, from which they pass immediately into the observation tube O, to flow finally out at the exit E.
(b). Arrangement of the jets J and K with regard to the mixing chamber.

introduced their continuous flow rapid reaction apparatus. To give concreteness to the description its application to the combination of O_2 with reduced haemoglobin will be taken as an example. In the apparatus shown in Fig. 2 the reagents, reduced haemoglobin and a

buffer solution equilibrated with known partial pressure of O_2, were forced by gas pressure from large earthenware bottles into a mixing chamber and thence into a glass observation tube about a metre long. The design of the mixing chamber was such that mixing was effectively completed in a period of less than a msec, that is within 3 mm or less of the beginning of the observation tube with the flow rates ordinarily employed. Thus during flow from the two bottles each portion of the observation tube was occupied by a mixture whose age increased with increasing distance from the mixing chamber. The time which had elapsed between mixing and observation of the mixture at any point

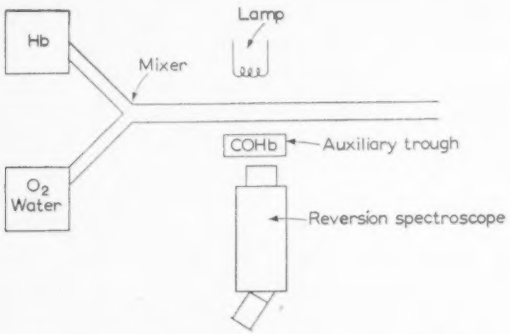

Fig. 3. General layout for HARTRIDGE and ROUGHTON's (1925) experiments on the combination of oxygen with haemoglobin. The reagents were contained in the pressure bottles and were driven by compressed nitrogen through the mixer to the observation tube. The proportions of COHb and O_2Hb in the combined system observation tube + auxiliary trough were obtained using a lamp and reversion spectroscope.

could be calculated from a knowledge of the flow rate and the cross sectional area of the observation tube. The saturation of the haemoglobin with O_2 was determined using HARTRIDGE's reversion spectroscope. This instrument measures very accurately the position of the centre of an absorption band and was originally introduced for determining the proportions of oxy- and carboxyhaemoglobin in mixtures, making use of the difference of some 60 Å in the positions of the α-bands of these compounds. In the present application the relatively much weaker spectrum of reduced haemoglobin is neglected, the amount of oxyhaemoglobin being determined by placing an auxiliary trough containing carboxyhaemoglobin between the light source and the observation tube. In these circumstances the progressive reaction of reduced haemoglobin with O_2 appears as a continuous shift in the apparent position of the α-band of the two-banded spectrum. The arrangement of the optical system is shown in Fig. 3.

This flow method was for long the only one by which information

about haemoglobin reactions in general could be obtained. Many and varied control experiments have been carried out with such apparatus and no doubt as to the general reliability of the data obtained with it exists. The drawbacks of the method are, first, that obtaining a sufficient rate of flow in haemoglobin solution of suitable concentration for visual spectroscopy requires the provision of large volumes of solution, HARTRIDGE and ROUGHTON using, in some experiments, volumes of reagents of the order of 10 l. to obtain a single kinetic curve. Second, the accuracy of the results is not very great since the total possible shift in the position of the absorption bands is always less than 60 Å and skill and experience in the use of the reversion spectroscope are essential to the successful use of the method. It is fair to say, however, that in every important instance the application of more modern methods of observation has confirmed the indications given by the visual procedure. In general the percentage saturation at a given time could usually be obtained to within about 5 per cent.

The next important development is due to MILLIKAN (1933a,b, 1936a) who used a photovoltaic cell and galvanometer to record the changes in light absorption due to the reaction. The short response time of his system led to substantial economy in reagents and the pressure bottles of HARTRIDGE and ROUGHTON'S apparatus were replaced by motor-driven syringes, while the higher sensitivity of his optical arrangements allowed the use of a capillary observation tube. He also introduced an ingenious differential arrangement using two photovoltaic cells connected in opposition to give first-order compensation for changes in lamp intensity or turbidity of the solutions being examined. The two photocells were placed behind filters so chosen that the reaction to be observed would produce a decrease in light transmitted by one filter and an increase in light transmitted by the other. The photocells and filters were then adjusted in relation to the observation tube so that each cell had the same output and the galvanometer deflection was nil. This adjustment was carried out with fully reacted mixture in the observation tube. A specially prepared "zero reacted" mixture was then introduced and the galvanometer deflection corresponding to the full extent of the reaction recorded. The motor-driven syringes containing the reagents were then allowed to discharge through the observation tube and the galvanometer deflection during discharge noted, giving the extent of the reaction at a definite time after mixing. By repeating this procedure at different rates of flow but at the same distance from the mixer a kinetic curve relating the extent of the reaction to time elapsed was obtained.

One weakness of this system is that unless a calibration curve is prepared the concentration of the reagents must be kept sufficiently low so that the change in light transmission due to the reaction is not

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more than 5 per cent at either of the wavelengths used in making the observation. This limitation arises because it is not possible to determine the contribution made by each photocell to the total galvanometer deflection and so to convert changes in percentage transmission to changes in optical density. Ideally, also, the system should be used only where it is quite certain that a single homogeneous reaction is

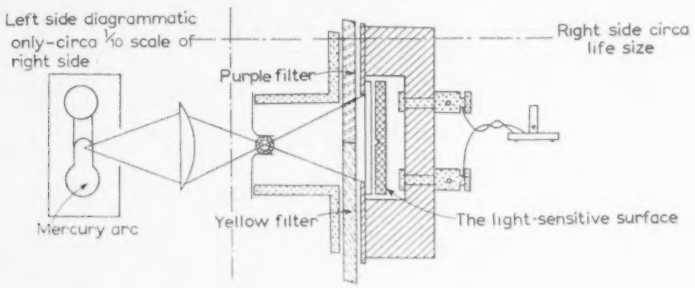

Fig. 4. Differential photo-colorimetric arrangement of MILLIKAN (1933a). The sensitive area of the photcell is divided into two by an insulating line forming a diameter, and the galvanometer is connected to the sensitive surface of the two halves. The cell then measures directly the difference in illumination of the two halves.

being observed and that side-reactions are fully excluded. A good example of difficulties of this kind is included in LEGGE and ROUGHTON's (1950) paper on the reaction of dithionite with sheep haemoglobin in solution and in the erythrocytes.

In an early paper describing the rapid flow method HARTRIDGE and ROUGHTON (1923a) pointed out that if the reaction had a half-time long compared with the time taken to set the reversion spectroscope there was no need to have continuous flow, and that the reaction could be followed by allowing mixture to enter the observation tube and then taking readings with the spectroscope at convenient time intervals. This suggestion, later given effect by ROUGHTON (1934c), forms the basis of the stopped-flow method developed first by CHANCE (1951) for studies on enzymes and adapted by GIBSON (1954) specifically for work with haemoglobin. In these later instruments the very rapid recording offered by the cathode-ray oscillograph was used, allowing quite rapid reactions to be observed and to be followed with great economy of fluid. This economy is due to the fact first, that flow need continue for a period of 10–20 msec only (as compared with 5 sec or so in MILLIKAN's apparatus), and second, that a whole kinetic curve covering the period from mixing up to any desired time thereafter can be recorded from a single portion of mixture (in the continuous flow systems a period of flow is needed for each single point on the kinetic

curve). In addition to economy the stopped flow method has the advantage that consecutive points on the kinetic curve are recorded using the same portion of mixture and are thus similarly affected by many of the errors which enter into measurements with flow apparatus. For example, if there is a small variation in temperature this variation has an opportunity to affect every point on a given kinetic curve; thus, although the accuracy with which a velocity constant can be determined is much the same with either method, the form of a kinetic curve can be much more precisely observed by the stopped flow method. It is also worth pointing out that the rate of working is much greater

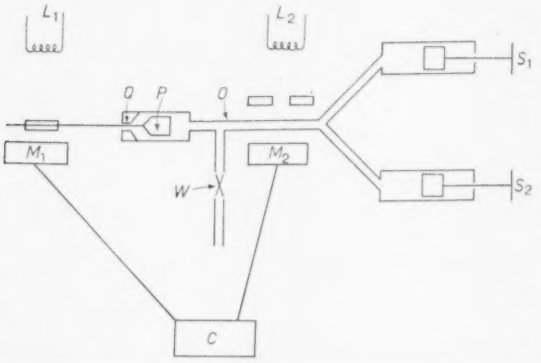

Fig. 5. General arrangement of stopped-flow apparatus. S_1, S_2, syringes containing reactant solutions; O, observation tube; PQ, flow-stopping device; W, tap to permit removal of spent reaction mixture; C, cathode-ray oscillograph; L_2, M_2, light source and photocell for observing the reaction; L_1 and M_1, light source and photocell for determining rate of flow of reagents (from GIBSON, 1954).

using the stopped flow method, with which, when set up, 10 replicate kinetic curves may be recorded photographically in 2 min.

The general lay-out of GIBSON's (1954) apparatus is shown in Fig. 5. Two points require comment: the light source L_1 and photomultiplier M_1 have the function of recording the movement of the piston P which is driven up to its stop Q by the flow of reagents from syringes S_1 and S_2. The flow rate prior to stopping can be obtained from this record. The second point concerns the stopping device Q which is particularly important not only because it causes the arrest of flow at a definite point in time, but because it stops flow sharply without previously slowing it down. If flow is allowed to stop gradually over a period of time, as in some of CHANCE's earlier designs, mixing becomes less efficient and the liquid at the point of observation when flow has finally ceased may be imperfectly mixed. This may result in a spuriously low reaction rate and also tends to produce unevenness in the

reaction record as streamers of imperfectly mixed fluid swirl in the path of the observation beam.

2. *Flash methods*

HALDANE and LORRAIN-SMITH discovered in 1897 that CO haemoglobin was sensitive to light, breaking down to give reduced haemoglobin and CO. The photosensitivity was only obvious when COHb was exposed to light in the presence of some other ligand able to compete with CO for combination with the photochemically formed reduced haemoglobin. Thus HALDANE and LORRAIN-SMITH observed the effect on exposing mixtures of oxy- and carboxy-haemoglobin to sunlight. Solutions containing mixtures of COHb and reduced haemoglobin are not obviously light-sensitive when exposed to ordinary laboratory light sources unless maintained in equilibrium with a large gas phase poor in CO. This is not, however, because an additional ligand other than CO is necessary for photosensitivity, but because when only CO is present the reduced haemoglobin formed by the action of light recombines quickly with it. If, however, a sufficiently brilliant light is applied to a solution of CO haemoglobin so that the rate of formation of Hb exceeds the rate of combination with CO, it is possible to bring about the conversion of COHb into reduced haemoglobin, and the reduced haemoglobin so formed will combine again with CO at the end of the illumination period. The high intensity light sources required have recently become available through the pioneer work of ALDINGTON (1948) in developing the electronic flash discharge tube. Under suitable conditions several hundred joules may be dissipated in a period of a few microseconds producing a flash of light corresponding to an instantaneous rate of some thousands of kilowatts. The electronic flash discharge tube has already been applied extensively in the field of physical chemistry by PORTER (see for example PORTER and NORRISH, 1954).

In the present application the flash method can be used to prepare mixtures of intermediate compounds of known composition. The work of BUECHER and NEGELEIN (1941–42) has shown that the quantum yield on irradiation of COHb and O_2Hb mixtures does not change with the composition of the mixture. That is, the 4 haems in the molecule are apparently independent of one another so far as photodissociation is concerned. Under these circumstances, as has been shown by GIBSON (1956a), the distribution of intermediates immediately following a brief flash of light will be given by successive terms in the expression $(p + q)^4$, where p represents the proportion of the total combined CO set free by the flash, and q that remaining in combination. Thus, the amount of the intermediate Hb_4 is p^4, of Hb_4CO, $4p^3q$, and so on.

The greatest difficulty in using flash methods is to be sure that the

reaction observed is not influenced by any kind of photochemical after-effect, and this constitutes a serious limitation to their use in problems where the results cannot be checked by comparison with flow methods. An example of what may be regarded as such an effect will be described later in this article.

A practical apparatus for applying the flash photolysis method to the haemoglobin reactions has been described by GIBSON (1956b). The general layout is shown in Fig. 6. The solution to be examined was placed in a cell C through which the observation light beam passed from the light source L on its way to the photomultiplier M, being rendered approximately monochromatic by interference filters. The

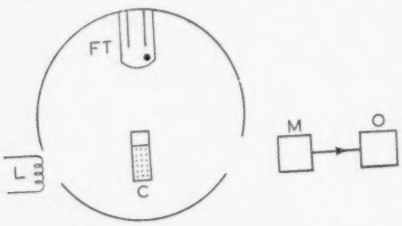

Fig. 6. Block diagram of flash photolysis apparatus. C, cell containing solution under examination; L, light source for recording the reaction; M, photomultiplier recording the reaction; O, cathode-ray oscillograph; FT, flash tube (from GIBSON, 1956b).

output of the photomultiplier was recorded with an oscillograph and camera, the oscillograph being triggered on firing the flash tube FT which brought about photodecomposition of the compound in C. The changes in optical density accompanying the return of the contents of C to their "dark" equilibrium position were thus obtained.

The possible field of utility of flash methods has recently been extended by AINSWORTH and GIBSON's (1957b) discovery that not only CO and cyanide (KEILIN and HARTREE, 1955) can be removed from haemoglobin photochemically, but also oxygen, nitric oxide and *iso*cyanides, though with lower quantum yields than in the first two cases.

IV. THE COMBINATION OF SHEEP HAEMOGLOBIN WITH CO

Although the reaction of haemoglobin with O_2 is physiologically more important than that with CO, the study of the CO reactions has always held a leading place in the development of knowledge about the kinetics of the gas reactions. This is because the reaction with CO takes place more slowly than the reaction with O_2, and further, the combination reaction is, relatively, very much more rapid than the dissociation reaction. The combination reaction can thus be studied

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alone without need to take into account the reverse reaction when interpreting the experimental results. The study of CO reactions has also been helped by exploitation of the photosensitivity of CO haemoglobin.

1. *The combination of reduced haemoglobin with* CO

The first observations were made in 1925 by ROUGHTON (1934a, b) who not only determined the approximate value of the velocity constant for the overall reaction, but also examined the effect of variation in the concentration of the reagents on the observed rate of reaction, which was first order with respect both to haemoglobin and CO. This result was of considerable theoretical importance because it showed that HILL's (1910) equation for the O_2 dissociation curve

$$y = Kp^n/(1 + Kp^n) \qquad \ldots.(13)$$

could not be regarded as possessing physical meaning, since the corresponding kinetic equation (14) would require the velocity to vary as the nth power of the CO concentration. As n has a value of about 2·5 the difference between the result actually observed and that predicted by HILL's equation would have been amply sufficient to be detected by ROUGHTON.

Although the results of ADAIR and SVEDBERG, already mentioned in the introduction, had shown that n in HILL's equation could not have the simple physical significance of representing the degree of aggregation of sub-units of mol. wt. 16,700, present in the haemoglobin molecule, they did not exclude the possibility that the four sub-units in the molecules might react simultaneously with CO in pairs or in threes, thus giving rise to the non-integral values of n required to fit the dissociation curves. ROUGHTON's result showed conclusively that simultaneous combination of several molecules of CO, as required by HILL's equation, did not occur. This experiment is shown in Fig. 7.

ROUGHTON (1934b) also examined the effect of pH and temperature on the overall reaction of CO with haemoglobin, finding that the temperature coefficient of the reaction was about 2 per 10°C and that altering the pH from 9 to 7 decreased the rate of combination about $1\frac{1}{2}$ times.

All these results were necessarily obtained using dilute solutions of haemoglobin. In 1935 BATEMAN and ROUGHTON carried out an important co-operative experiment with MILLIKAN, in which the combination of CO with reduced haemoglobin was followed optically by MILLIKAN using his new photoelectric apparatus and in the same solution by ROUGHTON's thermal method, in which the temperature change accompanying the combination reaction was measured using a thermocouple and galvanometer. Not only did the results of the two

experiments agree, showing that the optical and thermal changes went hand in hand, but both these new and more precise methods also showed marked deviations of the reaction from equation (6). These deviations which became progressively more pronounced towards the end of the reaction, consisted of the speeding up of the combination as the reaction proceeded. This result is, however, readily interpreted in terms of the intermediate compound hypothesis by which the reaction of gases with haemoglobin occurs in 4 steps. The acceleration can then be explained by supposing that after more than one molecule

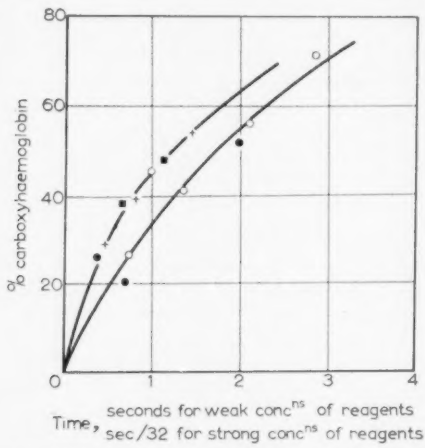

Fig. 7. Effect of thirty-two-fold variation in concentration of reagents upon rate of reaction CO + Hb → COHb at *p*H 7·4 and 10. (i) *p*H 10— ■ weak concentration; + strong concentration. (ii) *p*H 7·4— ● weak concentration; ⊗ strong concentration (from ROUGHTON, 1934).

of gas has combined with reduced haemoglobin, the combination of the remaining molecule or molecules takes place much faster.

The step from the recognition that ADAIR's hypothesis is applicable to the determination of numerical values of the individual velocity constants l'_1, l'_2, l'_3 and l'_4 is a very considerable one, for, as already pointed out, the fitting of 4 constants to a single kinetic curve can be achieved with a great number of sets of constants. In fact, trials with BATEMAN's (1910) solution for the case of constant [CO] and negligible back reaction suggested that to obtain a unique solution it would be necessary to know the percentage saturation of the haemoglobin with CO to within less than 0·1 per cent at any time after initiation of the reaction over a range of saturations up to 90 per cent, and that, even with such high precision, if one of the velocity constants were much greater than the others only the roughest indication of its size could be obtained.

This unpromising situation was, however, transformed by two new experimental factors. The first of these was the determination by ROUGHTON (1954) of the value of L_4, the equilibrium constant for the reaction $Hb_4(CO)_3 + CO \rightleftharpoons Hb_4(CO)_4$. The gasometric methods used were of such delicacy and precision that the percentage saturation of haemoglobin with CO could be determined over the range 98–100 per cent saturation to within 0·05 per cent saturation. Under these conditions the chief compounds present in appreciable concentration are $Hb_4(CO)_3$ and $Hb_4(CO)_4$ and the equilibrium constant L_4 can be determined by an extrapolation procedure. The dissociation velocity constant l_4 can also be found independently of the others by methods developed by ROUGHTON (1934c). It is thus possible, knowing l_4 and L_4 to calculate l'_4 and so to reduce the number of independently variable constants in BATEMAN's equation from 4 to 3. The result is a great reduction in the numbers of sets of constants which can be used to fit an individual reaction curve. It is possible to obtain values for the 3 constants l'_1, l'_2 and l'_3 describing the combination of the 3 molecules of CO with reduced haemoglobin with standard errors of about 10 per cent, provided that the percentage saturation of haemoglobin during the course of the reaction is known to within $\pm$ 0·3 per cent.

Even this reduced requirement of precision in knowledge of the form of the overall reaction curve exceeds by some 10 times the precision of measurement by MILLIKAN's (1936a) photoelectric continuous flow method. The new requirement was met, however, by adaptation of the stopped flow photo-electronic methods developed by CHANCE (1951) for varying rapid reactions. GIBSON (1954) has described a stopped-flow apparatus particularly intended for the study of the reaction of haemoglobin with gases with which reproducibility of individual reaction curves of the order of $\pm$ 1 per cent saturation could, under favourable conditions, be obtained, and in which the ease and speed of operation were such that 10 records might readily be averaged in the course of a single experiment, giving the percentage saturation at each time interval after mixing reduced haemoglobin with CO with a standard error of about the quantity required.

GIBSON and ROUGHTON (1957b) then made a systematic study of the combination of CO with reduced haemoglobin using the stopped-flow apparatus and having the help of Dr. DANIELS of the Cambridge Statistical Laboratory in fitting the observed curves with solutions of the kinetic equations obtained using the EDSAC digital computer. They did not use a wide range of conditions because of the very heavy arithmetical labours involved in fitting the curves, the total procedure involving many hours of work. Their principal results are summarized in Table 1 which shows the values of the first 3 combination velocity constants for CO at pH 9·1 and pH 7·1 at 20°C. This table shows

TABLE 1

Values of the Individual Velocity Constants l_1', l_2' *and* l_3' M^{-1} $sec^{-1} \times 10^{-3}$: *at* pH 7·1 *in Phosphate Buffer* 0·1 M, *at* pH 9·1 *in Borate Buffer* 0·05 M. *Sheep Haemoglobin* 0·025 mM: [CO] = 0·075 mM. 20°C

	l_1'	l_2'	l_3'
pH 7·1 . .	230	170	650
pH 9·1 . .	450	630	240

strikingly that change in pH does not only affect the absolute rate of reactions, but also the relative values of the 3 constants; thus at pH 9·1 l_2' shows an increase of 50 per cent over l_1', and l_3' is one-third of l_2'; at pH 7·1 l_1' and l_2' are roughly of the same order, but l_3' is much greater. This result is not compatible with the belief, long held, that the effect of pH on haemoglobin reactions can be formulated in terms of a scale-factor. Fig. 8 shows the details of one experiment giving the

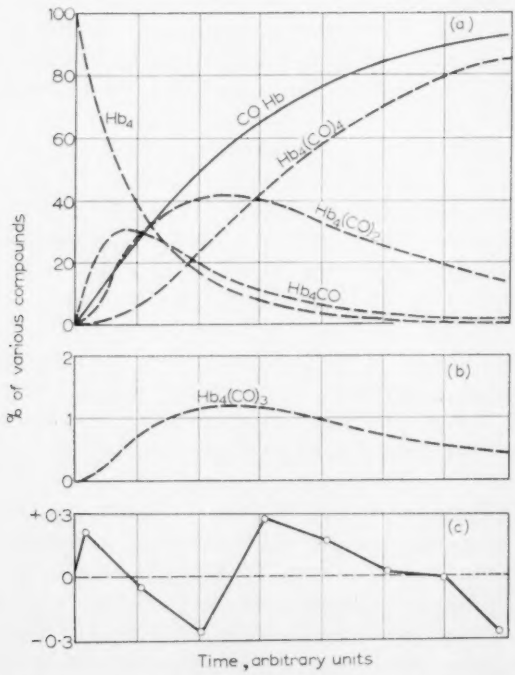

Fig. 8. A. Time course of the intermediates Hb_4, $Hb_4(CO)$, $Hb_4(CO)_2$ and $Hb(CO)_4$ and the total combined CO (i.e. percentage COHb for an experiment in which $l_1':l_2':l_3'::4:5{\cdot}9:2{\cdot}3$). B. Time course of the intermediate $Hb_4(CO)_3$. C. Discrepancy between calculated and observed values of percentage COHb (from GIBSON and ROUGHTON, 1957b).

concentrations of the different intermediates which should exist in the solution at varying times after mixing, and which illustrates also the agreement between the calculated and observed results obtained in many of the experiments. GIBSON and ROUGHTON were unable to find any change in shape of the reaction curves when the temperature of the reacting solutions was changed, the curves obtained with a temperature difference of 20°C being superposed very precisely by using a scale-factor (Fig. 9). It would appear, then, that the activation energy

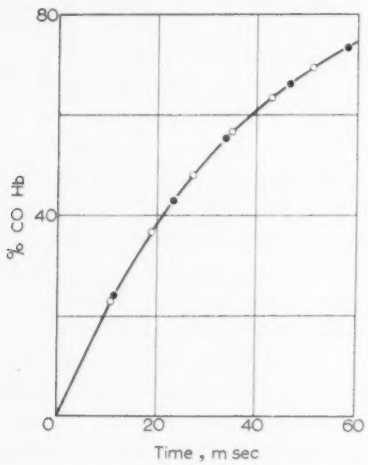

Fig. 9. The combination of 0·033 mM [Hb] with 0·099 mM [CO] measured at 3·1 and at 24·6°C, *p*H 9·1. The ordinate shows percentage COHb; the time scales for the two experiments have been chosen so that a length representing 1 msec for the 24·6°C experiment represents 4·03 msec for the 3·1°C experiment. 0, 24·6°C; ●, 3·1°C (from GIBSON and ROUGHTON, 1957b).

in each of the first 3 reactions is of the same order, the value being 10,500 cal. The value of l_1' and the activation energy are so related to one another as to give a reasonable value of the frequency factor in the usual rate-equation.

Although GIBSON and ROUGHTON thus succeeded in their aim of giving a complete description of the combination of CO with haemoglobin in terms of the 4 velocity constants, their methods are so laborious as to require the help of professional computers. It has recently become possible, however, to determine two of the velocity constants independently by the flash photolysis method. The procedure for obtaining l_4' has been described by GIBSON (1956a) and consists essentially of reducing the amount of light used until only a small proportion (p) of COHb is broken down by the flash. In these circumstances a solution may be prepared which contains effectively only $Hb_4(CO)_4$ and $Hb_4(CO)_3$. Thus for $p = 0{\cdot}05$, calculation shows that

immediately after the flash the solution should contain 81·45 per cent $Hb_4(CO)_4$, 17·15 per cent $Hb_4(CO)_3$, 1·3 per cent $Hb_4(CO)_2$, and only 0·05 per cent of other intermediates. Under these conditions, the initial rate of recombination of CO is:

$$-d[CO]/dt = l'_4[Hb_4(CO)_3][CO] \quad \ldots.(15)$$

and is thus a measure of the value of l'_4 substantially uncontaminated with other velocity constants. This method has recently been used by AINSWORTH and GIBSON (1957a) to follow changes in l'_4 over a wide range of pH and temperature.

It is reasonable to ask why it is necessary to use the flash method to prepare the mixtures of intermediate compounds, since it would seem at first sight that similar information could be obtained by mixing solutions containing COHb and Hb with excess CO in a flow apparatus. Such solutions must contain partially saturated intermediates similar to those produced photochemically, and the initial rate of combination of CO with the free gas binding groups would be expected to alter when the proportions of COHb and Hb initially present were varied. Experiments of this kind have already been reported by LEGGE and ROUGHTON (1950) who were unable to detect any difference between reduced haemoglobin and a COHb/Hb mixture containing 15 per cent COHb combining with CO. These experiments failed because L_4, determined by ROUGHTON (1954), is very much larger than the other intermediate equilibrium constants for CO. This means that equilibrium mixtures of COHb and Hb contain only small concentrations of $Hb_4(CO)_3$, the intermediate whose kinetic properties differ conspicuously from those of Hb_4 and the other intermediates. A second reason for the failure of LEGGE and ROUGHTON's experiment was that l'_4 is so great that with the concentration of CO (0·25 mM) used by them such $Hb_4(CO)_3$ as was present would have disappeared before the flowing mixture had reached the point of observation. The success of the flash method thus arises in part because it is possible to prepare mixtures of intermediates which have not reached equilibrium and in which the proportion of $Hb_4(CO)_3$ remains large.

The use of the flash method for preparing mixtures of intermediates is open to the objection that the calculation of the concentrations of the individual intermediate compounds rests upon several assumptions, not all of which can be fully justified. The validity of the method as applied in the determination of l'_4 has, however, been confirmed by experiments in which mixtures were prepared containing nitric oxide haemoglobin and carbon monoxide haemoglobin which were then subjected to flash photolysis. In a haemoglobin solution containing two ligands, but without free gas binding groups, GIBSON and ROUGHTON (1955a) have shown both theoretically and experimentally

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that the distributions of the intermediates Hb_4X_4, Hb_4X_3Y, $Hb_4X_2Y_2$, Hb_4XY_3 and Hb_4Y_4 are given by the binomial $(X + Y)^4$ where X represents the proportion of ligand x, and Y that of ligand y. In mixtures of COHb and NOHb, if the proportion of COHb is small, most of it will occur in molecules of the form $Hb_4(NO)_3CO$. On irradiation, these molecules will give rise to $Hb_4(NO)_3$ which may then be expected to combine once more with CO. The velocity constant of this combination, as has been shown by GIBSON and ROUGHTON (1955a) should depend only on the nature of the fourth ligand molecule and be independent of the nature of the 3 molecules already combined. Thus, after flash photolysis, the equivalent of equation (15) is

$$-\mathrm{d[CO]}/\mathrm{d}t = l_4'[\mathrm{Hb_4(NO)_3}][\mathrm{CO}] \qquad \ldots.(16)$$

allowing once more the determination of l_4'. The numerical values obtained in this way have shown good agreement with those obtained using COHb alone.

The flash method has also been applied by AINSWORTH and GIBSON (1957a) to the determination of l_1', which requires the isolation of the reaction

$$\mathrm{Hb_4 + CO \rightarrow Hb_4CO} \qquad \ldots.(17)$$

This can be done by observing the reaction between Hb and CO in its earliest stages where no appreciable amounts of intermediate compounds containing more than one molecule of CO have been formed. In practice, the rate constant may reasonably be calculated from observations covering the range 0–3 per cent saturation of haemoglobin with CO, the formation of $Hb_4(CO)_2$ being neglected. The error in the value of l_1' due to neglect of l_2' and higher constants depends on the relative sizes of l_1' and l_2', and for the special case where $l_1' : l_2' :: 4 : 6$ is shown in Table 2 which gives also the time course for the reaction for

TABLE 2

The Theoretical Course of the Combination of CO *with* Hb *where* $l_1' : l_2' : l_3' : l_4' :: 4 : 6 : 2 : 40$ *and* l_1' *has the value* $5{\cdot}48 \times 10^{-5}\ \mathrm{M^{-1}\ sec^{-1}}$

$[\mathrm{Hb}] + [\mathrm{COHb}] = 2{\cdot}58 \times 10^{-5}$ M; $[\mathrm{CO}] + [\mathrm{COHb}] = 2{\cdot}58 \times 10^{-6}$M

t (msec)	[Hb] (mM)	[CO] (mM)	$l_1' \times 10^{-5}$ *over interval* 0–*t*	*Error in* l_1' (%)	COHb (%)
0	0·02580	0·002580			
19	0·02563	0·002412	5·50	+ 0·4	0·65
38	0·02547	0·002252	5·58	+ 1·8	1·27
57	0·02532	0·002100	5·65	+ 3·1	1·86
76	0·02518	0·001958	5·70	+ 4·0	2·41
95	0·02504	0·001824	5·75	+ 4·9	2·93

a particular value of l_1' encountered in practice. It may be seen that even at the longest times used, the error in l_1' is not more than 5 per cent. This relation between the error in l_1' and the progress of the reaction is independent of the concentration of CO employed, and l_1' can be obtained from any measurement covering the initial stages of the reaction.

The advantage of the photochemical method is chiefly that the solutions are stationary during the determination, and the accurate

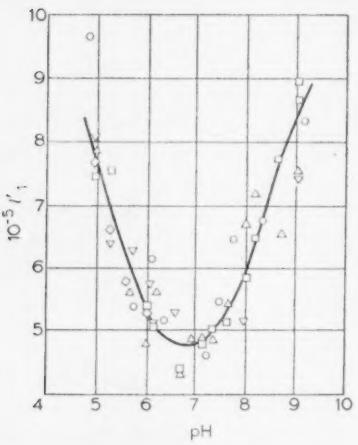

Fig. 10. The effect of *p*H on values of l_1' as determined by flash photolysis. 10 per cent COHb; 90 per cent Hb; total pigment 0·05 mM. The symbols of different shapes refer to experiments with blood from different sheep. 20°C.

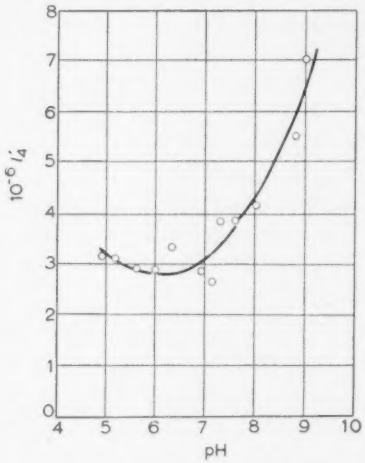

Fig. 11. The effect of *p*H on values of l_4' as determined by flash photolysis. Total pigment, 0·05 mM; 20°C.

recording of small optical density differences is thus made easier. In the practical determination of l_1' AINSWORTH and GIBSON worked with solutions containing 5–10 per cent COHb, the balance being reduced haemoglobin, and followed the reaction for periods of the order of 0·1 sec.

Some of the results obtained by AINSWORTH and GIBSON are shown in Figs. 10 and 11. The first of these shows the effect of *p*H on l_1'. This figure, while confirming the results already obtained by GIBSON and ROUGHTON (1957b) at *p*H 9·1 and *p*H 7·1 (though the average values found by AINSWORTH and GIBSON are rather higher than those obtained by GIBSON and ROUGHTON) adds the interesting new finding that there is a minimum in the *p*H velocity curve, the observed rate being least at the iso-electric point of reduced haemoglobin. Although a minimum has been observed in equilibrium work on the affinity of

O_2 for haemoglobin, no kinetic experiments have previously been carried out in detail over a range of pH values sufficiently wide to show the corresponding kinetic changes. In view of the considerable range of pH needed to show the minimum at pH 6·8 and the danger that the protein might have been adversely affected by the buffer, control experiments were carried out by the stopped-flow method in which the CO was dissolved in an acidic buffer and the haemoglobin diluted with water. In these circumstances, in which the acid buffer was allowed to come into contact with the haemoglobin for only a few msec before the determination was completed, the same rise in the combination velocity at acid pH was observed as shown in Fig. 11. It is believed therefore that the minimum represents a real property of the haemoglobin molecule related to the second oxygen-linked acid group described by Wyman (1949).

Similar experiments covering the same range of pH values have been carried out to determine the effect on l'_4. The results do not show a well marked minimum within the range studied. They show, however, a very considerable (and erratic) increase as the solution is made more alkaline than pH 8. These results again demonstrate clearly that the effect of pH on the overall reaction of CO with haemoglobin cannot be expressed in terms of a simple scale factor.

Ainsworth and Gibson (1957a) have also investigated the effect of the sulphydryl reagent p-chloromercuribenzoate (PCMB) on l'_1 and l'_4. They found an increase in l'_1 of about fourfold and a decrease in l'_4 of about twofold. These results are the kinetic equivalent of the effects observed by Riggs (1952) on the oxygen equilibrium curve. Riggs showed that the affinity of haemoglobin for O_2 was increased at low percentage saturations and decreased at high percentage saturations by the addition of PCMB to the solutions. It is clear that an analogous effect is operative in the case of CO.

One of the results with PCMB appears to contradict earlier work. Gibson and Roughton (1955a) examined the effect of PCMB on the overall reaction of CO with Hb and found only a small increase, which varied between 10 per cent and 40 per cent. This discrepancy may possibly be explained by extensive changes in l'_2 and l'_3, or may be due to a photochemical after-effect.

All the work so far described applies to dilute sheep blood solutions, which have been more extensively studied than the bloods of other species. One reason for this is that sheep haemoglobin has less tendency to split into sub-units on dilution than many kinds. Sheep blood is also relatively poor in methaemoglobin, a property particularly desirable in precise work near the bottom of the O_2 dissociation curve and in work on l'_1. A few observations have, however, been made on blood from other mammals. In all cases the overall reaction velocity is of

the same order and is influenced similarly by temperature and *p*H. Some results for human haemoglobin under physiological conditions have been given by GIBSON *et al.* (1955). As yet no studies of the time course of the combination reaction have been made which would give values of the individual intermediate constants, because of the very heavy work involved. Recent results of LYSTER (Ph.D. Thesis, Cambridge) on the dissociation curve of human and horse haemoglobins have suggested that the distribution of the equilibrium constants in these species differs very notably from that established by OTIS *et al.* (1955) for sheep haemoglobin, K_2 being much greater in both human and horse haemoglobin relatively to K_1 than in sheep. It is not known whether the kinetic consequences of this difference find expression in the combination or dissociation velocity constants, and studies on this point would be very desirable.

V. The Reactions of Myoglobin with CO

MILLIKAN (1936b) has shown that the combination of myoglobin with CO is second-order and has a rate constant of $3 \times 10^5\ M^{-1}\ sec^{-1}$ at room temperature. GIBSON (1956b) has recently confirmed MILLIKAN's results, using the flash method. Unlike haemoglobin, myoglobin does not show any variation in rate with change in *p*H.

All the haemoproteins so far discussed have had *proto*-haem for their prosthetic group. GIBSON and SMITH (1957) have described the preparation and properties of myoglobin reconstituted from the native protein and *proto*-haem, *meso*-haem, and *deutero*-haem. They found it possible to reconstitute myoglobin so that the velocity constants of the natural and reconstituted protein using *proto*-haem were the same. With *deutero*-haem the rate of combination with CO was more than twice that observed with *proto*-haem, thus changes in the side chain of the porphyrin nucleus are able, directly or indirectly, to exercise a considerable influence on the rate of reaction.

VI. Haemoglobins from Invertebrates

A few observations have been made on haemoglobins of invertebrates, some by GIBSON (1955) using the stopped-flow method and others by JONES and GIBSON (unpublished) by flash photolysis with the haemoglobins of *Cheironomus*, *Nephthys* and *Arenicola*. The results obtained are summarized in Table 3. Apart from *Cheironomus* the overall rate of reaction was similar to that observed with mammalian haemoglobins, but *Cheironomus* haemoglobin reacted a great deal faster. The use of the flash method has made possible a search for haem–haem interaction by decreasing the light energy used so as to produce only slight decomposition of the haemoglobin. Under these circumstances the velocity

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TABLE 3

The Combination of Various Haemoglobins with CO. *Velocity constants* M^{-1} $sec^{-1} \times 10^{-5}$ *at roughly Neutral* pH *in phosphate Buffers and Room Temperature*

Sheep (l') . .	1·2
Arenicola . .	2·9
Nephthys . .	4·0
Cheironomus . .	100
Earthworm . .	2·2

of recombination would be dominated by l'_n where n represents the number of haem groups forming a functional interacting unit in the molecule. It has been found that whereas *Arenicola* shows such interaction, *Nephthys* does not do so, although ultracentrifugal determinations have shown that *Nephthys* haemoglobin has a mol. wt. of 3×10^6 so each molecule must contain many haem groups. These, however, are arranged in some way which does not give rise to haem–haem interaction when they react with gas. The difference in numerical value of l'_n for *Arenicola* and l' for the overall constant for the same protein is a good deal less than that found for sheep haemoglobin, thus although interaction occurs it is quantitatively less striking than in the case of mammalian haemoglobin. The full development of comparative studies of different haemoglobins would require a very great effort, since information would be required not only about the rates of reaction with gases, but about the haemoglobins as chemical individuals. In view of the labour already applied to the study of mammalian haemoglobin and in particular sheep haemoglobin, such work appears scarcely practical at the present time and would require also quantities of material too great to be easily obtained from any but the larger species of animals.

A particular interest attaches to the haemoglobins of *Ascaris lumbricoides* which have been carefully studied by DAVENPORT (1949). Many of the properties of these haemoglobins are exactly the reverse of those associated with mammalian haemoglobin: thus, the dissociation of O_2 has a high temperature coefficient (5·1 per 10°C) and a half-time of the order of minutes rather than seconds. Reactions with CO have not been systematically investigated but GIBSON (unpublished) has found the combination velocity with CO of body-wall haemoglobin to be similar to that for mammalian haemoglobin, and, using ferricyanide to oxidize the free haemoglobin, found the half-time for dissociation of CO to be about 70 msec at room temperature. Thus, the reversal of roles between O_2 and CO is extended to cover the dissociation velocities of both ligands.

VII. Reactions with Oxygen

1. *The combination of sheep blood solutions with oxygen*

Information about oxygen is much less complete than that for CO, particularly in relation to the combination reactions and recent work has made it clear that the goal of describing the oxygen reactions in terms of the individual velocity constants may yet be some way off.

The first experiments on the combination of O_2 with reduced haemoglobin were carried out by Hartridge and Roughton (1925) who found that the reaction was rapid and that the velocity constants obtained by treating the reaction as a second order one varied a good

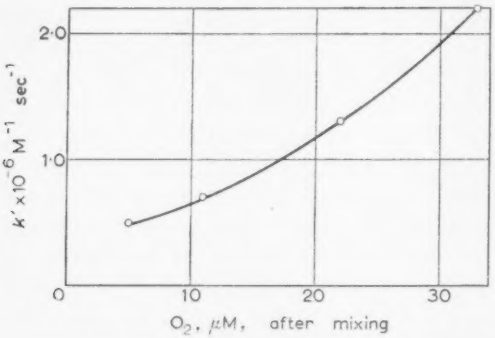

Fig. 12. The variation in the observed rate of the oxygen combination reaction when the concentration of oxygen is changed. The velocity constant k' is calculated assuming a second order reaction, and putting $k = 0$ in equation (6). [Hb] = 12·3 μM; borate buffer 0·05 M, pH 9·1; 20°C.

deal from one blood to another. In general they found a rate constant for the overall reaction (k') of about 3×10^6 M^{-1} sec^{-1} and that this value was little influenced either by temperature or the change in pH between 7 and 10. All these experiments were carried out at temperatures between 10 and 20° with haemoglobin concentrations of about 1×10^{-4}M. The conditions could not be widely varied because of the limitations of the reversion spectroscope, which was applied to the observation tube with an auxiliary trough of COHb interposed between it and the light source. Within the limits of experimental error and under the conditions just discussed, the reaction appeared to be approximately second order, and was described by equation (6).

These results of Hartridge and Roughton together with a few figures given by Gibson *et al.* (1955) have remained our only reliable source of information about the oxygen–haemoglobin reaction until very recently when Gibson and Roughton (unpublished) set about collecting more data, intending to apply, if possible, methods of analysis similar to those which had yielded successful results for the

Hb + CO reaction. When the oxygen concentration was varied it appeared that the initial rate constant, measured as near the beginning of the reaction as the stopped-flow method will allow, varied systematically with the oxygen concentration, rising rapidly as the latter was increased (Fig. 12). Furthermore, the rate constant, calculated as k' of equation (6) with k neglected, was never as great as in the experiments of HARTRIDGE and ROUGHTON, and at low oxygen concentrations was only from one-quarter to one-sixth as great.

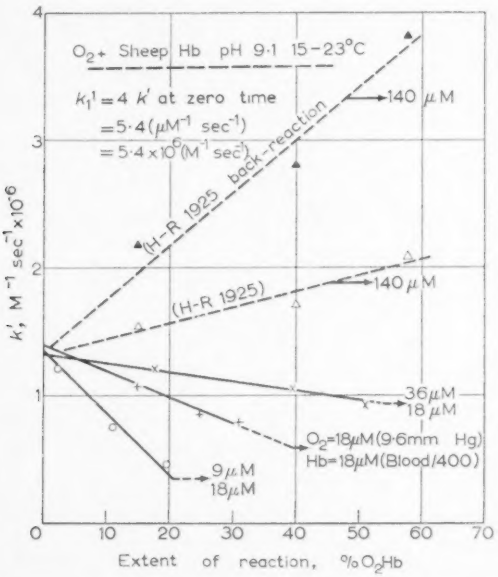

Fig. 13. The change in apparent velocity constant k' as the reaction of reduced haemoglobin with oxygen proceeds. H–R, 1925, is from data of HARTRIDGE and ROUGHTON (1925); other data from GIBSON and ROUGHTON, (1958).

These apparent contradictions seem to be due chiefly to neglect of the earliest stages of the reaction, which is unavoidable alike with the stopped-flow method and in HARTRIDGE and ROUGHTON's rapid flow method. If, instead of concentrating only on the earliest readings, the apparent rate constant is calculated over successive intervals of time covering the greater part of the reaction and plotted against the extent of the reaction, the results show the regularities of Fig. 13. At each concentration of oxygen there is a systematic alteration in apparent rate constant as the reaction proceeds, and extrapolation to zero reaction gives roughly the same initial rate constant at each concentration of oxygen used. Not only does this procedure restore internal consistency to the new stopped-flow observations but the original

observations of HARTRIDGE and ROUGHTON (1925) also fall into line when they are treated in the same way (shown in Fig. 13).

This somewhat unexpected result, which indicates that the apparent rate constant may increase, decrease, or remain the same as the reaction proceeds, seemed to call for some theoretical investigation and GIBSON and ROUGHTON have therefore obtained solutions to the set of equations (8) to (12) calculated by the EDSAC machine. The ratios and numerical values chosen for the constants can be regarded only as

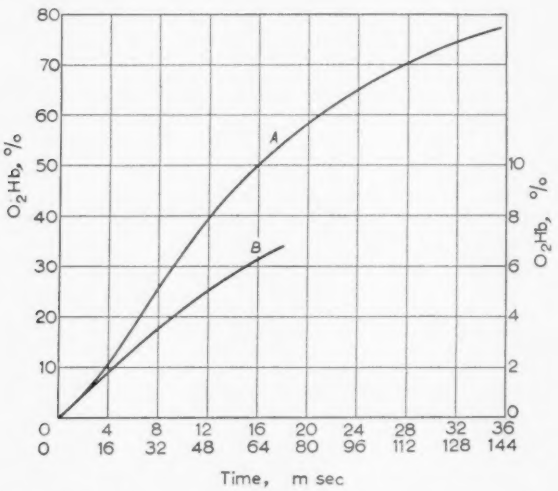

Fig. 14. The calculated course of the overall reaction in the combination of oxygen with haemoglobin with the velocity constants:

k_1' 2·1 × 10^6; k_2' 8·4 × 10^6; k_3' 12·6 × 10^6; k' 40 × 10^6 M^{-1} sec^{-1}
k_1 30; k_2 60; k_3 90; k_4 40 sec^{-1}
L.H. ordinate [Hb] = 19 μM; [O_2] = 38 μM. Upper time scale.
R.H. ordinate [Hb] = 19 μM; [O_2] = 4·75 μM. Lower time scale.

an example and may not be borne out by further work; nevertheless the results support the extrapolation procedure of Fig. 13 and deserve comment here. The ratios k_1'/k_1, k_2'/k_2, k_3'/k_3 and k_4'/k_4 were fixed by the work of OTIS *et al.* (1955); k_4 and hence k_4' were obtained from GIBSON and ROUGHTON (1955a), and k_1' chosen on the basis that at the lowest oxygen concentrations studied the initial rate calculated as k' of equation (6) should approximate to $k_1'/4$. It is known from the overall course of the deoxygenation reaction with dithionite that k_3 and k_2 must be somewhat larger than k_4, and, bearing this in mind, values were assigned in a rather arbitrary way to k_2' and k_3'. The numerical values are given in the legend to Fig. 14. The results of the computations showed, first, that with [Hb] = 19 μM and [O_2] = 38 μM the

apparent combination velocity constant k' increased rapidly as the reaction proceeded (Fig. 15, (I) curve $k = 0$); this is because the curve representing the overall reaction (A Fig. 14) is inflected. With a low concentration of oxygen (4·75 μM, B Fig. 14), the apparent velocity constant starts at the same value of $k'_1/4$ as in the case A of Fig. 14, but does not rise as the reaction proceeds. As the experimental observations at the higher oxygen concentrations do not include the first 20 per cent or so of the reaction, because of the dead-time of the stopped-flow apparatus, whereas almost all the reaction is recorded at the lower

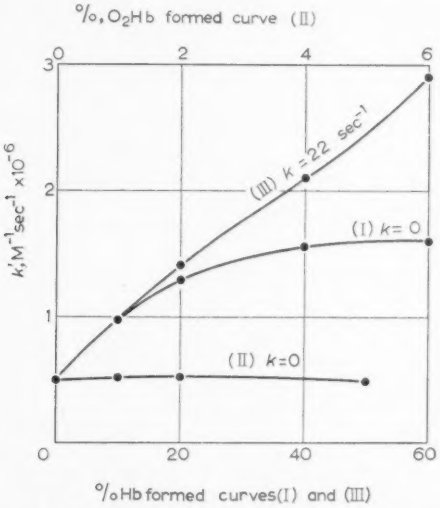

Fig. 15. The course of the apparent velocity constant k' as the reaction of reduced haemoglobin with oxygen proceeds. Data of Fig. 14.
(i) With [Hb] = 19 μM; [O_2] = 38 μM; k (of equation 6) = 0
(ii) With [Hb] = 19 μM; [O_2] = 4·75 μM; $k = 0$
(iii) With [Hb] = 19 μM; [O_2] = 38 μM; $k = 22$ sec^{-1}.

oxygen concentrations, the rise in the apparent velocity constant with oxygen concentration in the recent experiments is explained. The higher values found by HARTRIDGE and ROUGHTON (1925) as compared with the present experiments are due to the combination of two factors: first, the reversion spectroscope was not suitable for detecting saturations with oxygen below 30 per cent, so that the observations were limited to the range 30–65 per cent saturation and second, in applying equation (6) allowance was made for the back-reaction. The effect of such an allowance is shown in Fig. 15 in the curve $k = 22$ sec^{-1} and in the highest line of Fig. 13. The apparent combination velocity constant k' rises steeply as the reaction proceeds, to a value of twice that obtained when no allowance is made for back-reaction (Fig. 15, (I)

curve $k = 0$), thus explaining the discrepancy between the recent work of GIBSON and ROUGHTON and the original observations: it is found, in fact, that if HARTRIDGE and ROUGHTON's observations are recalculated on the same basis as the recent stopped-flow measurements there is excellent agreement between them. GIBSON and ROUGHTON (1958) have given a preliminary account of some of this work, in which they conclude that k_1' is about 5×10^6 M^{-1} sec^{-1} at *p*H 9·1 and 20°, or about one-half to one-third of the value previously thought likely.

2. *Determination of* k_4'

Although knowledge of oxygen combination constants is at present very incomplete, the velocity constant for the combination of the fourth molecule of oxygen has been obtained both directly and indirectly. The indirect method yields the constant by using K_4 from equilibrium measurements, (OTIS *et al.*, 1955), and k_4 obtained by kinetic methods to be described shortly. Another indirect method yields k_4' by determination of the ratio k_4' to l_4' from observation of the replacement reaction,

$$Hb_4(O_2)_4 + CO \rightarrow Hb_4(O_2)_3CO + O_2$$

under widely varying conditions. This, when combined with independent estimates of l_4' gives an estimate of k_4'. Further, it has been possible to determine k_4' directly by the method of flash photolysis, using three different means for producing the required intermediate Hb_4X_3, where X may be oxygen or CO. (1) Mixture of 90 per cent O_2Hb + 10 per cent COHb and moderate intensity light flash, (2) $Hb_4(CO)_4$ in presence of a low partial pressure of O_2 and low intensity light flash, (3) $Hb_4(O_2)_4$ high intensity light flash yielding $Hb_4(O_2)_3$. Determinations by these methods have yielded values of k_4' in reasonable agreement with one another and with the values obtained indirectly as just described. The value of k_4' under most conditions is about 3–4 times that of l_4', i.e. about 2×10^7 M^{-1} sec^{-1} at room temperature. It is particularly interesting that k_4' shows a temperature coefficient similar to that of l_4' whereas the overall reaction has repeatedly failed to show an obvious temperature coefficient either in flow or flash experiments. This wide difference in properties between the first and fourth molecules of oxygen to combine, points to an important change in the configuration of the haemoglobin molecule.

3. *Combination of oxygen with other pigments*

MILLIKAN (1936b) examined myoglobin finding that the rate was much higher than for mammalian haemoglobin with an average for k' of $1{\cdot}2 \times 10^7$ M^{-1} sec^{-1}. The rate was not influenced by change in *p*H; its temperature dependence was not investigated. GIBSON (1956a) has

confirmed MILLIKAN's result, and, in unpublished experiments both by the stopped-flow method and by flash photolysis, has obtained a temperature coefficient of 1·4 per 10°C. Making use of the recently discovered photosensitivity of oxymyoglobin (GIBSON and AINSWORTH, 1957b) and the short "dead-time" of the flash photolysis apparatus it has also been possible to show that the reaction between myoglobin and oxygen is accurately second order, a demonstration which, in view of the difficulties met with in work with mammalian haemoglobin, is worth noting (Fig. 16). These results were calculated using equation

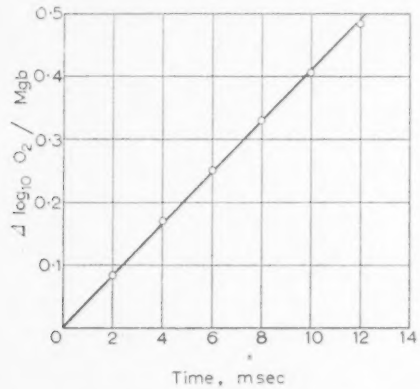

Fig. 16. Combination of myoglobin with oxygen. 0·1 M phosphate buffer *p*H 7·1; 1°C; 435 mμ. Total pigment = 13·1 × 10^{-6} M. Total oxygen dissolved and combined = 31·8 × 10^{-6} M. Flash photolysis breaking down 3·65 × 10^{-6} M O_2Mgb. $k' = 5 \times 10^6$ M^{-1} sec^{-1}.

(6) without allowance for the back-reaction, which, at the temperature of the experiment (1°C) has a very small value.

Another haemoprotein having a single group has been examined by GIBSON (1955b). This is the haemoglobin of the lamprey, whose equilibrium curve has been determined by WALD and RIGGS (1951). Unlike myoglobin (THEORELL, 1934) this compound shows a strong Bohr effect, the affinity decreasing markedly as the *p*H is reduced. GIBSON's observations showed that, again in contrast to mammalian haemoglobin, the main burden of accounting for the Bohr effect must be placed on the combination velocity constant, the dissociation rate measured with dithionite being unaffected by change in *p*H. The material is not easy to work with as the reduced pigment denatures readily, forming fibrous aggregates. The dissociation velocity constant is also unusually high (60 sec^{-1} at room temperature), so that the back reaction term must be taken into account in applying equation (6). Fig. 17 shows a comparison between the observed and calculated results at *p*H 6·2 and 9·1 for this pigment. The high combination rate

at *p*H 9·1 strained the capacity of the stopped-flow apparatus to its limit and called for the use of a narrow observation tube with a very small dead space between the mixer and the point of observation.

A few observations on the reaction with oxygen of the haemoglobins from *Arenicola marina* and the earthworm have been published by GIBSON (1955a). In view of the serious difficulties in interpreting kinetic curves where haem–haem interaction is present, it is sufficient to say

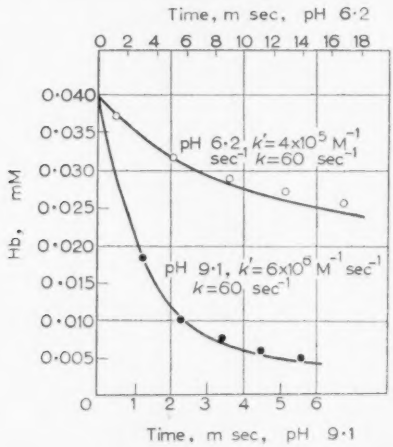

Fig. 17. Lamprey Haemoglobin combination of reduced haemoglobin with oxygen. [Hb] = 0·04 mM; [Hb] = 0·04 mM; (O_2) = 0·14 mM; 20°. k' = combination velocity constant; k = dissociation velocity constant.

that the overall rates were in both cases similar to those found for mammalian haemoglobin.

4. *Oxygen dissociation reaction*

The overall dissociation reaction was first studied by HARTRIDGE and ROUGHTON (1923b) who used dithionite to cause the reaction $HbO_2 \rightarrow Hb + O_2$ to proceed to completion. By ingenious and varied control experiments, they showed that the reaction did not proceed directly through the combination of dithionite with oxyhaemoglobin, but through the combination of dithionite with oxygen in physical solution leading to the withdrawal of oxygen from haemoglobin, and that the reaction of oxygen with dithionite is so rapid that no appreciable recombination of haemoglobin with oxygen can take place in its presence. The experiments of HARTRIDGE and ROUGHTON thus appeared to yield values for the overall reaction which could be used in calculations of physiological significance about the transfer of oxygen from the erythrocyte to the tissues. HARTRIDGE and ROUGHTON

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made measurements over a wide range of *p*H values and also examined the effect of temperature. They found that the rate of deoxygenation of haemoglobin increased rapidly as the solutions were made more acid (Fig. 18) the change being expressible in terms of a single oxylabile dissociation constant. Temperature accelerated the deoxygenation reaction, Q_{10} being 3·1 and the average value at 19° for k at *p*H 9·1, 12 sec^{-1}. The time course of the deoxygenation reaction, so far as

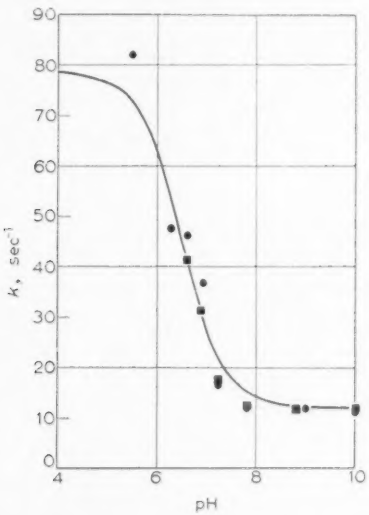

Fig. 18. The effect of *p*H on the rate of reduction of oxyhaemoglobin in the presence of dithionite (from HARTRIDGE and ROUGHTON, 1923b). Experiments were carried out with two different blood samples indicated with squares and circles.

HARTRIDGE and ROUGHTON could observe, was accurately first order. This condition would be met in terms of the intermediate compound hypothesis if *either* k_1, k_2 and k_3 were much greater than k_4 *or* if $k_4 : k_3 : k_2 : k_1 :: 4 : 3 : 2 : 1$.

More accurate observations of the deoxygenation reaction made by MILLIKAN (1933b) using his photoelectric apparatus, confirmed that the time course of the reaction, after an induction period during which the dithionite consumed oxygen initially present in physical solution, was substantially first order, but these covered only a rather restricted range of *p*H. LEGGE and ROUGHTON (1950) extended their observations to *p*H 7·1 using human haemoglobin, and observed an apparent plateau in the deoxygenation reaction at levels varying between 9 and 30 per cent saturation with oxygen. During the early part of the reaction the deoxygenation proceeded rapidly but slowed up strikingly towards

the end. This finding at first seemed to offer some prospect of evaluating the intermediate kinetic constants which would show up clearly in the special case where later constants are smaller than earlier ones. In these observations LEGGE and ROUGHTON were using the differential photo-colorimeter of MILLIKAN (1933a) in which changes in light absorption at two different wave-lengths are observed simultaneously, the outputs of the photo-cells being balanced one against the other so that with the fully reacted mixture present in the observation tube, there is a zero photo-cell output. The two spectral regions over which light absorption is measured are so chosen that when the reaction takes place an increase in optical density occurs at the one and a decrease at the other. With the connexion of the photo-cells employed, the two changes are additive in their effect on the total photo-cell output. This arrangement, which gives great stability and first-order correction for disturbances in light source output, is obviously not well adapted to the detailed study of spectral changes of the compounds being examined, and LEGGE and ROUGHTON soon found that the position of the plateau which they obtained was poorly reproducible and depended on the exact conditions under which the experiments were carried out. They found, moreover, that on studying the deoxygenation of haemoglobin within the erythrocyte, by mixing a red cell suspension with dithionite solution, no plateau could be seen under any conditions. Further, shaking the haemoglobin solution with oxygen before allowing it to react with dithionite raised the percentage saturation at which plateau effects were observed. LEGGE and ROUGHTON thus concluded that the plateau did not represent a true kinetic feature of the reaction of haemoglobin with oxygen, but must be due to a side reaction between oxidation products of dithionite and haemoglobin. This view was further supported by experiments carried out by GIBSON and ROUGHTON (unpublished) using the stopped-flow apparatus, when it was found that the apparent time course of the reaction varied considerably with the wave-length of light used for recording, and that after completion of the initial deoxygenation reaction which occupies about 0·2 sec at room temperature, slow drifts occur, which vary in extent and direction with the wave-length of the recording light. As a result of these experiments GIBSON and ROUGHTON concluded that the deoxygenation reaction as ordinarily carried out by mixing together dilute solutions of oxyhaemoglobin in equilibrium with room air (0·3 mM oxygen) and dithionite solutions could not yield results capable of bearing detailed kinetic analysis.

More recently, however, in the light of the detailed and careful studies carried out by DALZIEL and O'BRIEN (1957a,b) it has become quite clear that the disturbances of the deoxygenation reaction depend on the formation of hydrogen peroxide when dithionite reacts with

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oxygen, and that the extent of formation of the secondary compound (probably methaemoglobin hydrogen peroxide) is a function of the amount of oxygen in physical solution and the concentration of the haemoglobin itself. Using the studies of DALZIEL and O'BRIEN as a guide, experiments have been carried out in which the haemoglobin solutions have been equilibrated with 10 mm of oxygen and the dithionite solutions prepared with deoxygenated buffers in the absence of air. Under these circumstances it has been found possible to obtain

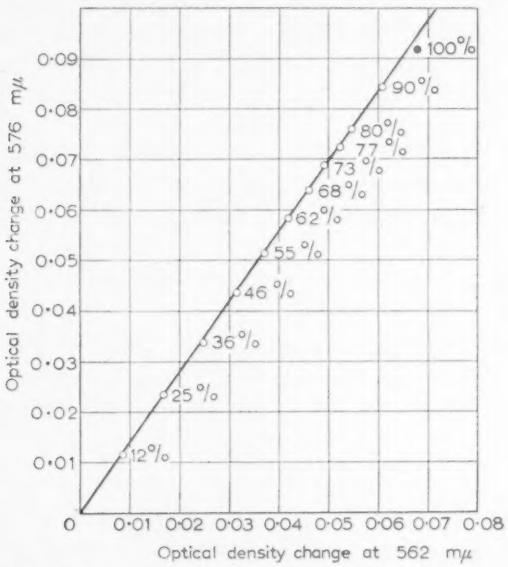

Fig. 19. Optical-density changes at intervals of 9 msec in a mixture of 0·08 mM oxyhaemoglobin and 0·4 per cent sodium dithionite at 576 mμ and 562 mμ. The changes are accurately proportional to one another except for the 100 per cent point, recorded some 30 sec after mixing which does not lie on the line.

reasonable agreement between the results at different wave-lengths, the discrepancy at its worst amounting to only 2 or 3 per cent. Further, the greater part of this discrepancy, as expected from DALZIEL and O'BRIEN's results, has been found to arise from reactions taking place after the main deoxygenation reaction is complete. Thus, if the optical density changes which have taken place in solutions of oxyhaemoglobin mixed with dithionite are recorded at definite time intervals after mixing, using light of more than one wave-length, and the optical density changes at one wave-length are plotted against those at the other, an excellent straight line results, while if the final reading some seconds after mixing is similarly plotted the resulting point does not lie on the straight line passing through the readings at intermediate times. This result is illustrated in Fig. 19. It is hoped, by making use

of the observation that the disturbing reactions are spread out through a comparatively long time, that some kinetic analysis of the overall reaction records may become possible.

It will be necessary, however, even when disturbances by hydrogen peroxide have been reduced as far as is possible, to proceed with great caution in the use of dithionite. Thus, at *p*H 7·1 GIBSON and ROUGHTON found that the overall reaction proceeded faster than the theoretical maximum set by the value of k_4 (which can be measured by several methods shortly to be described); k_4 necessarily limits the rate of the overall dissociation reaction, which, even in the case where k_3, k_2 and k_1 are all infinite, can only attain a numerical value equal to k_4. On several occasions, values of k which exceeded k_4 have been obtained which must be attributed, therefore, to a direct reaction between dithionite and oxyhaemoglobin at least at *p*H 7·1. It should be noted perhaps that HARTRIDGE and ROUGHTON's (1923b) original control experiments were carried out in more alkaline conditions.

5. *The velocity of dissociation of the first oxygen molecule from saturated sheep blood solutions*

When haemoglobin is in equilibrium with a mixture of oxygen and carbon monoxide at partial pressures such that there are few free gas-binding groups the partition of pigment between the two gases is given by

$$pz/qy = M \qquad \ldots.(22)$$

where $p = [O_2]$, $q = [CO]$, $y = [O_2Hb]$, $z = [COHb]$ and M is the partition coefficient. It is at first sight surprising that the equilibrium between oxygen and carbon monoxide can be so simply described when 5 species of mixed molecules of the form $Hb_4(O_2)_nCO_{4-n}$ are involved. The most obvious interpretation is that the affinity of a given haem for oxygen and carbon monoxide does not change when its neighbours in the haemoglobin molecule exchange oxygen for carbon monoxide or vice versa. ROUGHTON (1954) has introduced the necessary notation to deal with this situation, and has deduced the relations between the equilibrium constants for the various species of mixed molecules.

The most natural kinetic interpretation of equation (22) and of ROUGHTON's deductions from it is that the rate of combination of a ligand molecule with a molecule of haemoglobin having one free gas-binding group depends only on the nature of the molecule combining and not on the nature of the ligand molecules already combined. The corollary is that the rate of dissociation of a ligand molecule from fully saturated haemoglobin depends only on the nature of the dissociating molecule and is independent of the nature of the ligand molecules occupying the three remaining places in the haemoglobin molecule.

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If these kinetic suppositions are correct, the rate of displacement of one ligand by another can be made to depend on one kinetic constant only. For example, if oxyhaemoglobin were mixed with a solution of carbon monoxide at a partial pressure so high that the chance of a newly formed free gas-binding group reacting with an oxygen molecule instead of a carbon monoxide molecule was negligible, the rate of disappearance of oxyhaemoglobin would, at first, be given by

$$-\mathrm{d}[\mathrm{Hb_4O_8}]/\mathrm{d}t = k_4[\mathrm{Hb_4O_8}] \qquad \ldots.(23)$$

so that under these conditions the initial rate of replacement of oxygen by carbon monoxide would be a measure of k_4. On the principle that the rate of dissociation of a given ligand is independent of the nature of the other molecules in combination, however, the rate of replacement should continue to be governed by k_4 throughout the reaction, and so allow this constant to be measured independently of the other dissociation velocity constants. It is clear, further, that the same general principle applies to pairs of ligands other than oxygen and carbon monoxide, the rate of displacement being determined by the rate of dissociation of the ligand displaced.

In practice, it is not always possible to arrange the partial pressures of the gases so that the chance of a newly formed free gas-binding group recombining with oxygen is negligible. It is then necessary to allow for the reverse reaction as follows:

writing $[\mathrm{O_2Hb}] = y$, $[\mathrm{O_2}] = p$, $[\mathrm{Hb}] = 1 - y$, $[\mathrm{CO}] = q$,

$$-\mathrm{d}y/\mathrm{d}t = ky - k'p(1 - y) \qquad \ldots.(24)$$

$$\mathrm{d}(1 - y)/\mathrm{d}t = ky - k'p(1 - y) - l'q(1 - y) = 0 \qquad \ldots.(25)$$

since it is justifiable to regard the rate of change of concentration of free gas-binding groups as negligible by comparison with the changes in oxyhaemoglobin concentration. On substitution from (25) in (24)

$$-\mathrm{d}y/\mathrm{d}t = ky\{l'q/(k'p + l'q)\} \qquad \ldots.(26)$$

the observed rate of replacement of oxygen by carbon monoxide expressed as a rate constant is then

$$(-\mathrm{d}y/\mathrm{d}t)/y = r = kl'q/(k'p + l'q) \qquad \ldots.(27)$$

which may be re-written

$$\frac{1}{r} = \frac{1}{k}\left[1 + \frac{k'p}{l'q}\right] \qquad \ldots.(28)$$

Inspection of (28) shows that if k, k', l' and q are regarded as constants, $1/r$ should vary linearly with p, the intercept on the ordinate being

$1/k$ and the slope $k'/kl'q$. The treatment just given applies to a haemoprotein having only one gas-binding group; a full derivation has been given by GIBSON and ROUGHTON (1955a) who have shown that for mammalian haemoglobin the equivalent of (28) is

$$\frac{1}{r} = \frac{4(1 + k'_4[O_2]/l'_4[CO])}{k_4} \qquad \ldots\ldots(29)$$

the intercept when $1/r$ is plotted against $[O_2]$ now being $4/k_4$, and the slope $4k'_4/k_4l'_4[CO]$.

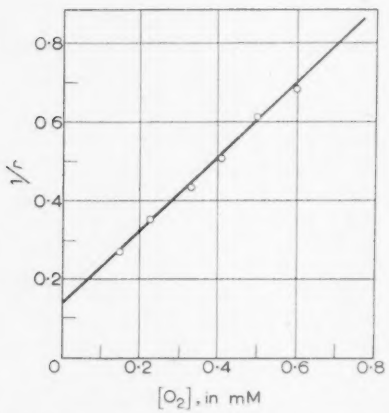

Fig. 20. Determination of k_4 from rate of displacement of O_2 by CO at different O_2 concentrations. [CO] = 0·51 mM; [O_2Hb] = 0·095 mM in all cases; temperature = 18·5°C; *p*H 9·1, borate buffer 0·025 M. r = observed rate of replacement of O_2. Ordinate $1/r$; abscissa [O_2] in mM. The linear relation between $1/r$ and [O_2] is in good agreement with equation (29).

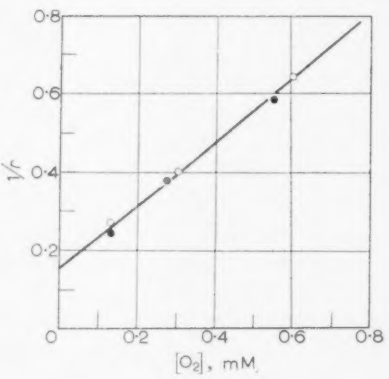

Fig. 21. Determination of k_4 with (i) [O_2Hb] 0·1 mM, (ii) 0·1 mM [O_2Hb] + 0·2 mM [COHb]; *p*H 9·1 borate buffer 0·025 M; 19°C; [CO] = 0·50 mM throughout. The agreement between $1/r$ in the two cases supports the belief that the dissociation velocity constants are independent of the nature of the ligand molecules remaining in combination.

Equation (29) has been thoroughly tested in numerous control experiments, two of the most important of which are illustrated in Figs. 20 and 21. The first of these shows that a linear relation does exist when $1/r$ is plotted against [O_2], while Fig. 21 shows that the same value of k_4 is obtained when the displacement reaction is started by mixing 100 per cent O_2Hb with CO, and by mixing 33 per cent O_2Hb:66 per cent COHb with CO. In the second case there is only 1·3 per cent Hb_4O_8 present initially and almost the whole of the reaction observed corresponds to the dissociation of oxygen from haemoglobin molecules containing both oxygen and carbon monoxide; thus the agreement shown in Fig. 21 provides a powerful confirmation

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of the belief that the dissociation velocity constants are independent of the nature of the ligand molecules remaining in combination.

Some of the results of GIBSON and ROUGHTON (1955a) are summarized in Table 4. It is interesting to note that a change in *p*H from 7·1 to 9·1 is apparently without effect on k_4, a result which contrasts markedly with the findings of HARTRIDGE and ROUGHTON (1923b) in

TABLE 4

The Effect of pH on k_4 and k_4'/l_4'; 19°C

Sheep			
*p*H	5·9	7·1	9·1
k_4 (sec^{-1})	79	30	30
k_4'/l_4'	6·9	2·8	3·3
Man			
*p*H		7·1	9·1
k_4 (sec^{-1})		26	24
k_4'/l_4'		3·0	4·1

their experiments on the effect of *p*H on the deoxygenation of haemoglobin in the presence of dithionite. On the other hand, the energy of activation, 18·4 kcal, is similar to that found by the earlier authors (22·1 kcal). The numerical value of k_4 is about $1{\cdot}5k$: if $k_4:k_3:k_2:k_1 :: 4:3:2:1$, the limiting case corresponding to NO haem–haem interaction, $k_4 = 4k$. If $k_1 = k_2 = k_3 = \infty$, corresponding to infinite haem–haem interaction, $k_4 = k$, which is nearer to the figure actually observed than the first result. It is clear, therefore, that some at least of the lower intermediate constants exceed the statistical values considerably. It would seem that in the dissociation reaction the situation is parallel to that described for combination, the reaction accelerating as it proceeds. It follows that the overall haem–haem interaction, which finds its expression in the ratios of the equilibrium constants, is contributed to both by the combination and dissociation velocity constants.

VIII. THE DISSOCIATION OF CO

The information available about this reaction is confined to a knowledge of the velocity of the reaction

$$Hb_4(CO)_4 \rightarrow Hb_4(CO)_3 + CO$$

This limitation arises because of the lack of any reagent which combines with CO in solution but does not injure the protein. It is thus impossible to remove the CO liberated in the reaction, and because of the very high affinity of CO for haemoglobin the effect of mixing COHb even with a very large volume of deaerated buffer is slight, scarcely any dissociation occurring. ROUGHTON (1934c) has reported the results of some experiments in which reduced haemoglobin arising from the dissociation of COHb was removed from the reaction mixture by the addition of ferricyanide which oxidizes reduced haemoglobin rapidly to methaemoglobin. He found, however, that the rate of disappearance of COHb in solution in the presence of ferricyanide was about 50 per cent faster than would have been expected from the results of gas displacement experiments which yield l_4. The discrepancy is probably due to the formation of intermediate compounds in which some of the iron atoms have been oxidized and some remain in the reduced form. These mixed molecules appear to give up CO more rapidly than the fully saturated ones.

In principle the ideal method for determining the velocity of dissociation of CO from fully saturated haemoglobin would be to use CO molecules labelled with ^{14}C. If haemoglobin were equilibrated with ^{14}CO, exchange of labelled and unlabelled molecules would take place at a rate determined by l_4. The difficulty lies in separating the CO in physical solution from that in combination after the exchange reaction has been allowed to proceed for a definite period.

The method has, however, been used by GIBSON and ROUGHTON (1952) who shook a solution of $^{14}COHb$ in a tonometer containing CO in the gas phase and drained off the liquid phase at the end of the shaking period. The rate of the reaction was calculated from the amount of radioactivity which had been transferred to the gas phase during the shaking period. It is difficult to obtain fully satisfactory results by this method because at high total pigment concentrations equilibrium between the liquid and gas phases cannot be established sufficiently rapidly by vigorous shaking and at low pigment concentrations corrections for CO in physical solution become large. The results of a considerable number of experiments, however, have yielded results strongly supporting those obtained in replacement reactions involving ligands other than CO.

The first experiments on the dissociation of CO were carried out by ROUGHTON (1934c) who shook a small volume of a dilute solution of COHb vigorously with a large volume of gas containing a high proportion of O_2. Under these conditions the CO set free by the dissociation of COHb largely escapes into the gas phase and the reverse reaction is not prominent. He also carried out all-fluid experiments with O_2.

More recently GIBSON and ROUGHTON (1957c) have made use of NO

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as displacing reagent instead of O_2. This has practical advantages because NOHb is so much more stable than COHb that it is easy to arrange for the equilibrium to be displaced in the direction of very extensive formation of NOHb. COHb is itself sufficiently stable to

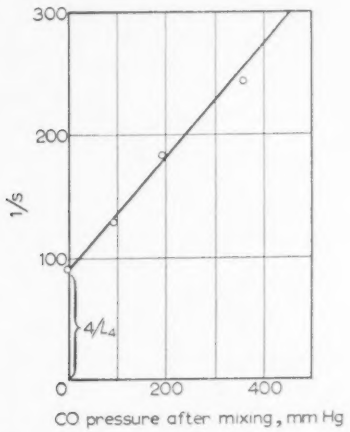

Fig. 22. Determination of l_4 by replacement of CO by NO. Test of equation (29). Spectrophotometric observation of reaction; sheep blood diluted 1/200; 0·05 M, borate buffer *p*H 9·1; 20°C; [NO] = 6·75 × 10^{-6} M; $j_4''/l_4' = 4{\cdot}0$.

allow a neat demonstration of the validity of the general theory put forward for determining k_4, l_4 and j_4 and in particular of (29). This test is illustrated in Fig. 22, where it is possible to determine the rate of replacement of CO by NO at a partial pressure of CO so low that the back-reaction is quite negligible, and the first point in the figure is

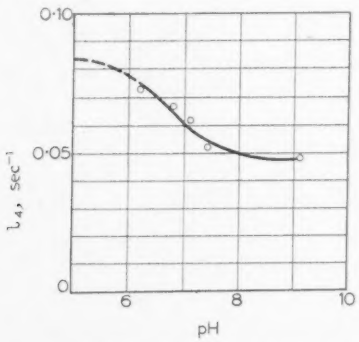

Fig. 23. The curve shows the effect of *p*H on l_4 over the *p*H range 6·0–9·0 at 23·5C. The curve is calculated on the assumption that l_4 for the ionized form is 0·084 and for the unionized form 0·048. The corresponding values at 19° would be 0·051 and 0·029, which are close to the general means found with several samples of sheep blood.

virtually "on" the ordinate. This point corresponds satisfactorily, as would be expected, with that obtained by an extrapolation experiment of the usual kind, shown in the same figure.

Using the NO replacement method GIBSON and ROUGHTON (1957c) have determined the value of l_4 and the effect on it of variation in pH over the range of 6–9. Their results are summarized in Fig. 23. The

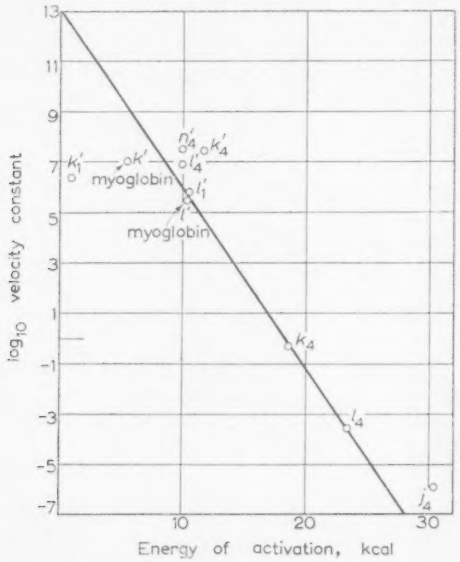

Fig. 24. The relation between energy of activation and velocity constant for several haemoglobin–ligand reactions.

temperature coefficient of l_4 was determined by ROUGHTON (1934c). His value of about 4 for Q_{10} has been confirmed by GIBSON and ROUGHTON (1957c), who have pointed out that the velocity constant and the activation energy of the reaction, like that for k_4 and j_4, give a normal value to the frequency factor (Fig. 24).

IX. THE DISSOCIATION OF NO FROM $Hb_4(NO)_4$

The principles applied in studying the dissociation of carboxyhaemoglobin and oxyhaemoglobin can be transferred directly to the case of nitric oxide haemoglobin. In practice, the displacing agent must be carbon monoxide both because of the very high affinity of nitric oxide for haemoglobin and because prolonged exposure of nitric oxide haemoglobin to air leads to destruction of the pigment with the formation of a brown precipitate. The reaction takes place slowly, the half-time at room temperature and pH 9·1 being about 6 hr, and in applying

the shaking method (Gibson and Roughton, 1957c) it is sufficient to rotate the tonometer gently in a water-bath. The temperature coefficient of the reaction is unusually high at 5·5 per 10°C, a value which, however, gives a value for the frequency factor in good agreement with those found for the dissociation of oxyhaemoglobin and carboxy-

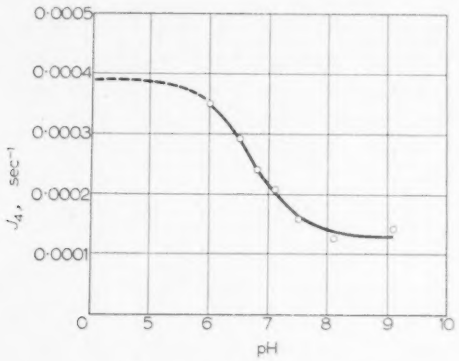

Fig. 25. The effect of pH on j_4.

haemoglobin (Fig. 24). Change in pH of the medium has the usual effect on the rate of dissociation and this is larger than the effect on COHb (Fig. 25).

X. The Combination of NO with Haemoglobin

The only experiments have been described by Gibson and Roughton (1957a). The observed reaction is about 10 times faster than that found with oxygen and so lies on the limit of the application of the stopped-flow method. No detailed study of the kinetics is possible, and it is not clear whether anomalies such as those met in the oxygen reaction apply to nitric oxide also. The reaction appears to have a moderate temperature coefficient of about 1·4 per 10°C, and to be faster at pH 9·1 than at 7·1. The only constant which has been estimated alone is j_4', which can be obtained from j_4, L_4, and N, the ratio between L_4 and J_4, and from l_4' using the ratio j_4'/l_4'. Estimates made in this way have been given by Gibson and Roughton (1957c), the product of J_4 and j_4 yielding $4{\cdot}9 \times 10^7$ M^{-1} sec^{-1} at pH 9·1 and 19°. Alternatively the data of the experiment shown in Fig. 22 give j_4'/l_4' as 4·0. If l_4' is taken as 8×10^6 M^{-1} sec^{-1}, j_4' is estimated as $3{\cdot}2 \times 10^7$ M^{-1} sec^{-1}. In view of the large number of different measurements and the range of methods used in reaching these estimates, the discrepancy does not lie outside the limits of possible error. A slip has occurred in the calculations of Gibson and Roughton (1957c), j_4 being taken as

$1{\cdot}07 \times 10^{-3}$ instead of $1{\cdot}07 \times 10^{-4}$; consequently j_4' is given as $4{\cdot}9 \times 10^{8}$ M^{-1} sec^{-1} in place of $4{\cdot}9 \times 10^{7}$ M^{-1} sec^{-1} as above.

XI. The Effect of Temperature on the Reactions of Haemoglobin

Although some mention of this important characteristic of the gas reactions has been made throughout the paper, the collected results are of sufficient interest to receive special attention in a separate section. The rate of a chemical reaction is represented in standard physical chemical theory by the equation

$$k = \frac{RT}{Nh} e^{\Delta S^*/R} e^{-\Delta H^*/RT} \quad \ldots\ldots(30)$$

where k is the velocity constant in sec^{-1} or M^{-1} sec^{-1}, S^* is the entropy of activation, H^* is the heat of activation, N is Avogadro's number, h Planck's constant, R the gas constant and T the absolute temperature. The term $\frac{RT}{Nh} e^{\Delta S^*/R}$, called the frequency factor, often has a value of the order of 10^{13} for unimolecular reactions and 10^{10}–10^{11} for bimolecular reactions; these numbers offer a convenient definition of the normality of a reaction, and allow one reaction to be compared with another. The information at present available about the haemoglobin gas reactions has been summarized in Fig. 24, where the line has been drawn on the assumption that $\Delta S^* = 0$, i.e. about 100 times the ordinary rate for a bimolecular reaction. This line shows the expected value for second-order reactions with varying energies of activation. Many of the haemoglobin gas reactions appear to be approximately normal by this standard: thus the dissociation velocity constants for O_2, CO and NO, which differ a million-fold, all give points close to the line, as do l_1', and l' for myoglobin. There is, however, a striking contrast between O_2 and CO, k_1' being about 1,000,000 times smaller than theory predicts, though k' for myoglobin is only about 100 times smaller than expected.

Deviations in the opposite sense are shown by the combination velocity constants for the fourth molecule of each of the ligands O_2, CO and NO, and are evidence of the large configurational differences between reduced and saturated haemoglobins, reflected in the widely different ease of access of the first and fourth molecules of oxygen.

In Fig. 24 only one line has been drawn to represent both combination and dissociation reactions: at first sight, two lines should be needed, one for the second-order combination reactions, the other for the dissociations, which are first-order. In fact, as Haurowitz (1951)

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has shown, the usual formulation of the dissociation reactions is incomplete, as they should be written:

$$Hb_4X_4 + H_2O \rightleftharpoons Hb_4X_3{\cdot}H_2O + X \qquad \ldots.(31)$$

Under ordinary circumstances these interesting observations have no kinetic consequences, since the concentration of water (56 M) may be regarded as constant throughout the reaction. The points in Fig. 24 have, however, been calculated taking account of equation (31).

XII. Some Anomalies and Miscellaneous Observations on Haemoglobin Kinetics

So far the results presented have been dealt with in simple terms, supposing that the combination of each species of haemoglobin intermediate with a ligand follows a second-order equation and that its dissociation is accurately first-order. Although this appears, as has been seen, to be true of many of the reactions studied, under some conditions new effects appear which cannot be satisfactorily described in this simple way.

Another limitation to the study of haemoglobin kinetics arises from the implicit assumption that there is only one species of haemoglobin molecule present in the diluted blood of a single animal. It is now known that this is not so, and that in sheep blood, with which so much work has been done, it is possible to show that more than one electrophoretically distinct haemoglobin may be present in some animals. It is not known how far these electrophoretic differences are reflected in a difference in kinetic properties, but if there were two species of haemoglobin with notably different properties, detailed analysis of the kind undertaken by Gibson and Roughton (1957b) in determining the values of the successive constants for the combination of carbon monoxide might be open to serious objection.

Another assumption is that the extinction coefficients of the different intermediate compounds are the same, so that from a spectrophotometric point of view a mixture of reduced and oxygenated haemoglobin can be treated as if only two compounds, Hb and HbO_2, were present. Although several comparisons between percentage saturation determined gasometrically and optically have been made without any evidence of differences between the intermediate compounds appearing, few of the experiments were really suited for detecting them as the comparisons were made over the middle range of percentage saturation. Calculation shows, however, that there is little change in the concentrations of the different intermediates in the middle range of saturations, as haem–haem interaction (expressed in the large value of K_4 as compared with K_1, K_2 and K_3) results in the main compounds present

being Hb_4 and Hb_4O_8. It is interesting in this connexion that JUNG (1957) has stated that the time course of the formation of azide-methaemoglobin appears to be different when the wave-length of the observing light is varied. Unhappily, few experimental details have been published and the weight to be attached to these experiments is uncertain. It is not clear how nearly monochromatic were the observation beams or how great were the optical-density changes measured. If the light used were insufficiently homogeneous, the optical-density changes considerable, and the wave-lengths selected at a point in the spectrum where the optical density of either methaemoglobin or azide-methaemoglobin is changing rapidly with wave-length, the observed effects could readily be explained without recourse to varying extinction coefficients for the intermediate compounds. From the theoretical point of view, such changes seem unlikely since the visible absorption bands correspond to electronic transitions involving 40–80 kcal. The energies involved in haem–haem interaction are much smaller than this, and need not produce any significant change in the absorption spectrum. For the present, then, it seems reasonable to assume that the extinction coefficients of the various intermediates are proportional to the numbers of gas-binding groups occupied.

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XIII. FURTHER COMMENTS ON THE OXYGEN COMBINATION REACTION

ROUGHTON (1934d) described some experiments on what he called quick and slow equilibria between oxygen and haemoglobin. In these, dilute solutions of sheep blood containing about 90 per cent O_2Hb were mixed with oxygen-free buffers and the mixture observed (*a*) 0·5 sec later, (*b*) 2 min later. The interval of 0·5 sec allows ample time for the dissociation of oxygen following the reduction of oxygen tension to become complete, and on the basis of the velocity constants for the oxygen reactions which have been measured no change would be expected after 0·5 sec. When ROUGHTON used a buffer at *p*H 10, he could not detect any change between 0·5 sec and 2 min, but with phosphate buffers of *p*H 6·3 and acetate buffers at *p*H 6·5 and 5·0 large differences were observed; in one example the quick equilibrium at *p*H 6·3 with phosphate buffer was at 45 per cent O_2Hb and the slow equilibrium at less than 20 per cent O_2Hb.

Using the stopped-flow apparatus GIBSON and ROUGHTON (unpublished) have observed similar drifts following on the oxygen combination reaction where this is carried out with concentrations of oxygen such that only partial saturation is obtained at equilibrium. An example of this kind at *p*H 9·1 is shown in Fig. 26, where it is clear that about one-quarter of the observed total change in optical density

has a half-time of about 1 sec, while the initial quick change is half-completed in less than 0·1 sec. This effect has not yet been systematically investigated, but it seems likely that even greater changes than those shown in Fig. 26 will be found at *p*H 7·1. It is difficult to discuss these anomalous results adequately, and it is not yet clear whether they are likely to be found with more concentrated solutions of haemoglobin than those so far employed. One attractive hypothesis can, however, be dismissed on theoretical grounds; it is that at a time of, say 0·1 sec

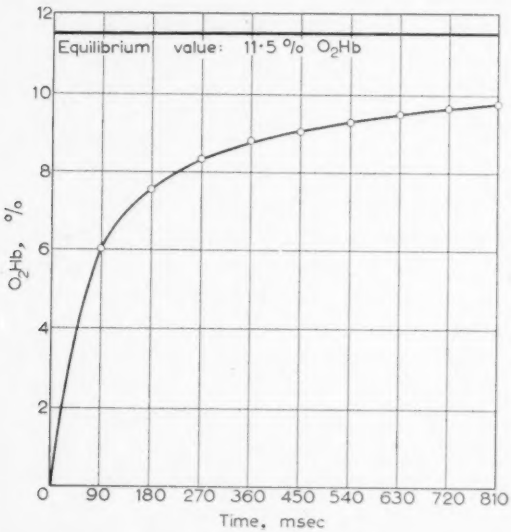

Fig. 26. Quick and slow reactions of oxygen with haemoglobin. 0·02 mM sheep Hb; 0·0047 mM O_2; 21·5°C; borate buffer *p*H 9·1; $\lambda = 432$ mμ.

after mixing the proportions of the various intermediate compounds are chiefly determined by the values of the first 2 combination constants, while at equilibrium it is determined solely by the 4 equilibrium constants. It might thus be possible for slow changes to take place while the kinetically determined distribution of intermediates was replaced by the equilibrium one. If this were so, however, it would appear in the results of the EDSAC computations which should also show the slow drift phenomenon of Fig. 26. No result of this kind has so far been obtained.

This phenomenon may perhaps be related to the marked changes which take place in the haemoglobin molecule on combination with gases. Thus, it is known that the solubility and other properties of the molecule also change. It is tempting to suppose that this extensive rearrangement requires an appreciable time for its completion resulting in the difference between the quick and slow equilibria.

If this is so, the phenomenon should occur not only with dilute solutions in the presence of oxygen but with more concentrated haemoglobin solutions and with other ligands. The experimental investigation of the problem is likely to be difficult, as with ligands having a higher affinity than oxygen the amount present in physical solution when quick equilibrium has been established will not be great enough to allow a significant change in percentage saturation when the affinity of the haemoglobin changes. The difficulty is accentuated with strong solutions of haemoglobin, and, even in the case of oxygen, it may be necessary to measure the amount of oxygen remaining in physical solution rather than the percentage saturation of the haemoglobin.

XIV. A Reactive Form of Haemoglobin

When Gibson (1956b) applied the method of flash photolysis to the reactions of myoglobin with oxygen and carbon monoxide it was hoped that it could be used to study the kinetics of the combination of oxygen with reduced haemoglobin, preparing known mixtures of intermediate compounds and measuring the initial rate of combination with oxygen. When the appropriate experiments were carried out, however, anomalous results were obtained.

At first the simple flash apparatus of Gibson (1956a) was used, in which a commercial flash tube dissipated 100 J with a time constant of about 150 μsec. A mixture of O_2Hb and COHb in equilibrium with a gas mixture containing a small proportion of CO in the desired partial pressure of O_2 was prepared: on firing the photolysis flash, it was expected that CO would be removed from combination from the haemoglobin and that O_2 would replace it, the rate of replacement being determined by the various intermediate oxygen combination velocity constants (which are believed to be much larger than the combination velocity constants for CO). After oxygen had combined with the haemoglobin it would gradually be replaced by CO which would remain in combination with it, in virtue of the very small dissociation velocity constant of COHb as compared with that for O_2Hb. It should then be possible to repeat the flash and go through the cycle once again. In practice, in experiments carried out at pH 9·1 using an interference filter to give approximately monochromatic light, the optical-density changes were always much smaller than expected, and the reaction observed appeared to have a negative temperature coefficient. Furthermore, the optical-density changes were greater at low temperatures than at room temperature. In view of these curious results, and because of the difficulty of interpreting optical-density changes observed using interference filters, the matter was not taken

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further at the time, but was deferred until the completion of a new flash apparatus of higher power which could dissipate 600 J with a time constant of about 20 μsec. It was hoped that this apparatus would strip the CO from the haemoglobin molecule rapidly enough to avoid interference from possible very rapid reactions between oxygen or CO and partly saturated haemoglobin molecules. With this apparatus, which used also a monochromator instead of interference filters, the earlier results were substantially confirmed. The optical-density excursion continued to be less than that calculated from the extinction coefficients of the solutions used and there was again a negative temperature coefficient for the optical-density excursion and the initial rate of optical-density change at 430 mμ (isosbestic point for COHb and O_2Hb). With the monochromator it was possible to confirm that the oxygen was exchanged for CO on firing the flash, the optical-density changes at 420 mμ (absorption maximum for COHb) being in good agreement with expectation. The velocity constant of the part of the reaction which was observed at room temperature agreed well with the figures given by HARTRIDGE and ROUGHTON (1925) for solutions of high haemoglobin and oxygen concentration.

Following on these results, experiments were next carried out with carboxyhaemoglobin alone at *p*H 9·1, using high partial pressures of CO, in order to obtain a fast reaction comparable with the rates expected for the oxygen reaction. At 20° and *p*H 9·1 it was now found that the optical-density change (O.D.C.) at 430 mμ on flashing was less than that expected from the concentration of the solutions. The O.D.C. could be increased to the theoretical value, by reducing the CO concentration, and on careful inspection of the reaction records at the lower CO concentrations, a small initial rapid recombination reaction could be seen. On cooling the solutions to 3° and repeating these experiments, it became clear that the failure to observe the expected O.D.C. was due to the formation of a rapidly reacting compound which persisted in the solutions at 3° for a few msec after flashing. At moderate CO partial pressures a well marked biphasic reaction (Figs. 27 and 28) was seen, a rapid O.D.C. taking place in the first 3 or 4 msec after photolysis. Under suitable conditions this rapid phase could be made to account for as much as 80 per cent of the total observed O.D.C. At 3° and low CO concentration only a small part of the reaction took place rapidly, so that the rapidly reacting form apparently reverts to ordinary reduced haemoglobin in a brief space of time (Fig. 27). Assuming that the COHb formed by the combination of the rapidly reacting form with CO has the same absorption spectrum as normal COHb, the absorption spectrum of the rapidly reacting form can be obtained. It appears to resemble ordinary reduced haemoglobin, but the Soret maximum is displaced by about 2 mμ towards the red.

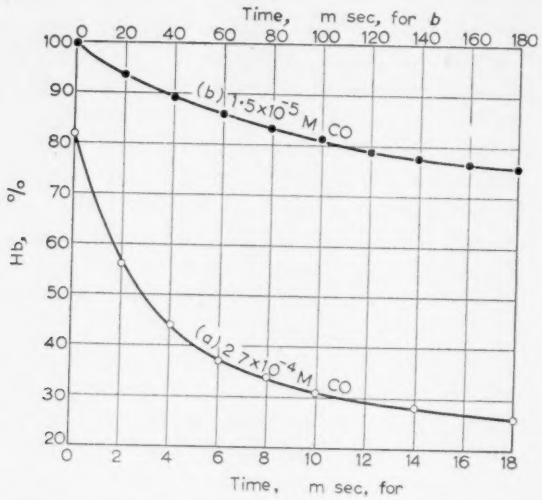

Fig. 27. The reaction of CO with Hb. [Hb] = $1{\cdot}1 \times 10^{-5}$ M; (*a*) [CO] = $2{\cdot}7 \times 10^{-4}$ M, (*b*) $1{\cdot}5 \times 10^{-5}$ M; 0·025 M borate buffer *p*H 9·1; 3°C; flash photolysis 600J.

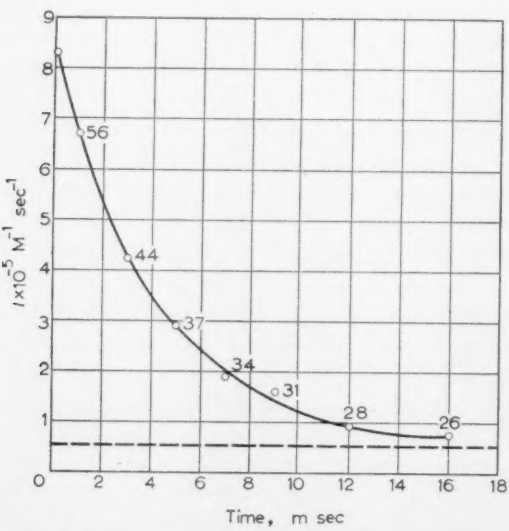

Fig. 28. ○ Time course of the velocity constant for the combination of CO with Hb. [Hb] = $1{\cdot}1 \times 10^{-5}$ M; [CO] = $2{\cdot}7 \times 10^{-4}$ M; 0·025 M borate buffer *p*H 9·1; 3°C; flash photolysis 600J; observation beam 435 mμ. Figures on curve give the percentage Hb remaining at each time interval. Dotted line gives expected course of reaction if no rapidly reacting form were present.

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A further repetition of the experiments with oxygen at low temperature and at relatively low oxygen concentrations showed that an analogous phenomenon could be observed with it also, an initial abnormally rapid phase, lasting for a few msec, being seen. This phenomenon of the transient existence of a reactive form of reduced haemoglobin seems to explain the difficulties encountered in attempting to apply flash photolysis to the oxygen reaction.

It has not yet been possible to carry out a full series of flash photolysis experiments but it has been observed that the appearance of the reactive form of haemoglobin is more evident at *p*H 9·1 than at *p*H 7·1. At the latter *p*H, indeed, it is only just possible to discover an irregularity in the reaction records, and the optical-density changes at *p*H 7·1 are in much better agreement with expectation than those at *p*H 9·1. It is thus possible that some use may yet be made of flash photolysis in studying the reaction with oxygen at *p*H values less than 9·1. Little use can be made of the reaction records where there are unobserved very rapid portions, because under these conditions the proportions of intermediate compounds present when observation begins are unknown and there does not seem, at present, to be any way of calculating the composition of the mixture of intermediates produced as a result of the rapid reaction.

The origin and significance of these results is not yet clear, nor is it known if rapidly reacting forms of reduced haemoglobin appear when dissociation of the ligand molecule takes place spontaneously rather than as a result of photochemical action. The effect is qualitatively similar to some early observations of ROUGHTON (1934d) which indicated that when oxyhaemoglobin is reduced by dithionite the haemoglobin formed will combine with carbon monoxide faster during the first 0·2 sec or so after reduction than if it is allowed to stand for a few seconds before mixing with the second ligand. These findings could be obtained only under quite sharply defined conditions of *p*H and temperature, being found at *p*H 10 and room temperature, but were absent at *p*H 10 and 33°, and at all the temperatures examined at *p*H 6·6. Its relation to the photochemical phenomenon is obscure, the time-scale in the two cases differing by several orders of magnitude. It is tempting to suppose that the rapidly reacting form of haemoglobin and the "quick" and "slow" equilibria just discussed have a common origin in the reorganization of the molecule which is associated with haem–haem interaction. On such a basis the rapidly reacting form of reduced haemoglobin would be regarded as the normal product of dissociation of a haemoglobin–ligand complex whose properties are made accessible by flash photolysis, rather than as an abnormal product arising by photochemical action, which has no relation to the ordinary reactions of haemoglobin with gases within the body.

Although at present all discussion of these matters is highly speculative, it is worth describing two further anomalies which may be related to the rapidly reacting haemoglobin effect. As described in relation to the dissociation of the first ligand molecule it is possible from equation (29) to obtain the ratio k'_4/l'_4 in experiments on the displacement of oxygen from haemoglobin by carbon monoxide. Analogous experiments with myoglobin give the ratio k'/l', which, unlike the value for haemoglobin, may also be determined directly by measuring k' and l' separately. In direct determinations the ratio k'/l' has been found in several experiments to be about 30 (in agreement with MILLIKAN, 1936b), while displacement experiments on a number of preparations have given an average value of about 10. This discrepancy lies far outside experimental error, and has not so far been satisfactorily explained. Another way of confirming the existence of this discrepancy is by allowing reduced myoglobin to react simultaneously with a mixture of oxygen and carbon monoxide. Experiments of this kind carried out with the flow apparatus have confirmed the ratio of about 30 for k'/l'. It seems possible that this puzzling discrepancy may be associated with the transient existence of a rapidly reacting form of reduced myoglobin for which the ratio k'/l' is lower than for the stable reduced myoglobin. Such an effect cannot, however, be a general one, for GIBSON (1955b) found that values of k'/l' for the haemoglobin of the lamprey as determined directly and from displacement experiments were in good agreement.

A further example of an anomalous result which may be related to the occurrence of a rapidly reacting form of haemoglobin has been found by AINSWORTH and GIBSON (1957a) in studying the effect of *p*-chloromercuribenzoate on l'_1 by the flash method. On adding the mercurial there was a 4 to 8-fold increase, whereas GIBSON and ROUGHTON (1955a) reported an increase in l' of only 10–20 per cent, using a stopped-flow apparatus. It seems possible that this difference may be due to longer persistence of the rapidly reacting form when the —SH groups of the protein are blocked.

XV. CONCLUSION

The kinetics of the reactions of haemoglobin with gases have now been under active investigation for some 30 years. At almost any time during that period it would have been possible for a reader not actively engaged in the field to conclude that final and definitive description of these reactions was at hand. In the event, the application of new technical methods, while confirming the accuracy of the experimental observations which have gone before, has revealed new difficulties and complexities even in apparently simple situations. It is perhaps fair to say

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that we now know enough about the kinetics of the reactions of gases with haemoglobin to deal with the physiological problems of oxygen transport in the circulation, and that further work is being undertaken in order to find out more about the reactions for their own sake. This is desirable because haemoglobin may well be taken as a model for the action of enzymes, and the complexities met with in working with it may prove a pointer to the much greater complications likely to appear when the study of enzyme kinetics can be carried to similar lengths.

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DIFFUSION AND SIMULTANEOUS CHEMICAL REACTION VELOCITY IN HAEMOGLOBIN SOLUTIONS AND RED CELL SUSPENSIONS

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DIFFUSION AND SIMULTANEOUS CHEMICAL REACTION VELOCITY IN HAEMOGLOBIN SOLUTIONS AND RED CELL SUSPENSIONS

F. J. W. Roughton

I. Introduction

Just over thirty years ago, Hartridge and Roughton (1927) reported the first *in vitro* measurements of the rates of uptake of oxygen and carbon monoxide by suspensions of sheep red blood corpuscles. A brief recapitulation and discussion of some of this early work* will, it is hoped, provide a helpful—and reasonably simple—introduction to the problems to be considered in the present article.

For their studies of the kinetics of entrance of dissolved gases into red blood corpuscles (red cells for short) Hartridge and Roughton made use of the rapid reaction velocity method (Hartridge and Roughton, 1923a) which they had already applied to the kinetics of the reactions of oxygen with haemoglobin in homogeneous solution (Hartridge and Roughton, 1923b; 1925). Their procedure in the case of red cell suspensions was as follows:

(*a*) One part of completely deoxygenated sheep's blood was mixed with about 50 parts of gas-free 1 per cent NaCl solution, the resultant blood cell suspension being prevented from settling by frequent agitation.

(*b*) In a second container, a suitable volume of 1 per cent NaCl solution was equilibrated with a gas mixture containing either oxygen at partial pressures ranging from 100 to 760 mm Hg, or carbon monoxide within a similar range of partial pressures.

(*c*) The two fluids were driven by compressed gas from their respective containers into the mixing chamber of the rapid reaction apparatus. Thence the mixed suspension travelled down a glass observation tube, wherein the average percentage saturation of the red cells with oxygen (or carbon monoxide) was determined by the Hartridge Reversion Spectroscope at a series of points along the observation tube, the fluid being kept in constant motion throughout the spectroscopic observations. The time corresponding to each measured percentage saturation of the red cells was readily calculated from the rate of fluid flow and

* The main findings of Hartridge and Roughton have been fully confirmed by recent and more accurate work with modern photo-electronic methods.

the distance of the point of observation from the mixing chamber; with six or so different points of observation a reasonably complete curve for the kinetics of the process could be obtained.

For further details of the rapid reaction velocity technique, the reader should consult the original papers of HARTRIDGE and ROUGHTON or WEISSBERGER's (1953) *Technique of Organic Chemistry*, Vol. VIII, Chapter X (Rates and Mechanisms of Reactions).

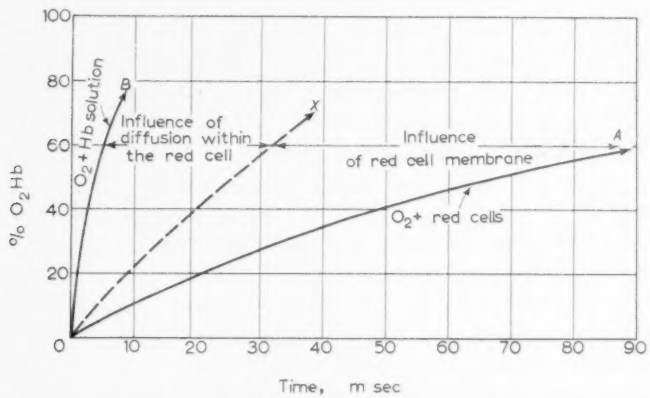

Fig. 1. Comparison of rate of uptake of oxygen by a red cell suspension of sheep blood (a) with the rate of uptake of oxygen by sheep haemoglobin in homogeneous solution. The broken line curve (X) is the calculated rate of uptake of oxygen by a membraneless layer of haemoglobin solution of the same concentration as in the red cell and of thickness equal to the average thickness of the red cell.

Fig. 1 gives the results of a typical experiment, at 15°C, on the rate of uptake of oxygen by:

A. A sheep red cell suspension (whole blood 1/100).

B. Homogeneous sheep haemoglobin solution of the same gas combining capacity as that of the red cell suspension in *A*.

The concentration of the dissolved oxygen in the mixed fluid at the start was the same in both cases, viz. $1{\cdot}25 \times 10^{-3}$ M. The time to reach 40 per cent saturation is seen to be about 20 times longer in the red cell suspension (*A*) than in the homogeneous haemoglobin solution (*B*). The difference is found to be due to two factors:

1. The resistance offered by the red cell membrane to the passage of dissolved oxygen.

2. The finite thickness of the interior of the red cell. The first oxygen molecules to pass through the cell membrane tend to combine with haemoglobin in the outermost layers of the red cell, thus opposing a barrier of partially combined haemoglobin to the next oxygen molecules to enter the red cell. The overall rate of combination of oxygen with haemoglobin in the red cell is thus progressively slowed

down by a diffusion factor as well as by the normal Law of Mass Action factor, the latter arising of course, from the fall in the concentration of the reacting species as the reaction proceeds.

The relative effect of these two factors could be readily appraised if observations could also be made on the rate of uptake of oxygen by a layer of solution, containing haemoglobin at the same concentration as in the interior of the red cell, and the same average thickness as the red cell, but with *no* bounding membrane. Such observations have not yet proved technically possible, but the problem can be solved approximately by the theoretical methods to be described later in this article, if the rate constants of the chemical reaction of oxygen with haemoglobin inside the red cell and the diffusion coefficient of oxygen within the latter are both known. The broken-line curve, Fig. 1, X, gives the results of such calculation in the present case. The space between Fig. 1, A and Fig. 1, X is clearly an index of the red cell membrane resistance, whereas the space between Fig. 1, X and Fig. 1, B shows up the effect of joint diffusion plus chemical reaction within a finite layer of haemoglobin solution, as compared with the rate of the homogeneous chemical reaction of oxygen with haemoglobin.

The main objectives of the present article are:

1. To summarize the work done on the development of theoretical solutions, both by analytical and by numerical approximation methods, for the uptake of oxygen, carbon monoxide and nitric oxide in flat layers and in spheres of haemoglobin solution, with or without bounding membranes.

2. To discuss the application of this theoretical treatment to the various experimental observations of these processes in thin layers of haemoglobin solution *in vitro* and also—and especially—in intact red cell suspensions, leading in the latter case not only to an evaluation of the joint influence of diffusion and chemical reactions within the interior of the red cell, but also to an estimation of the actual permeability of the red cell membrane to O_2, CO and NO.

The reason for the limitation to O_2, CO and NO is that it is only for these three substances that any adequate experimental data are available for diffusion plus chemical reaction in haemoglobin-containing systems. It is hoped, however, that the methods and results in these particular cases may serve as a guide for a much wider range of biological processes in which diffusion and chemical reaction velocity are jointly limiting factors, as and when other examples of this type come to attract similar detailed investigation.

The solution of diffusion problems, or of diffusion plus chemical reaction problems, depends very much on the particular geometry of the system under consideration. It is usual to treat of layers, cylinders and spheres, but—as already mentioned—the present methods, in the

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case of haemoglobin, have so far been mainly limited to layers and in lesser degree to spheres. Cylindrical diffusion, though of immense importance in other biological systems such as nerve fibres, has so far found little application in blood problems.

There are two fortunate circumstances which help greatly in the study of diffusion plus chemical reaction of O_2, CO and NO in haemoglobin solutions of the high concentration found in the interior of mammalian red cells, i.e. 30 per cent (w/v)* and upwards:

1. There is good reason to suppose that the purely chemical kinetics of the haemoglobin reactions with these dissolved gases are not only qualitatively, but also quantitatively, much the same in 30 per cent (w/v) haemoglobin solutions as in the much more dilute solutions, of the order of 0·1 to 0·2 per cent (w/v), which have mainly been studied up to the present.

2. The haemoglobin molecules are so closely packed that their translatory motion is practically nil (PERUTZ, 1948). Consequently no allowance need be made for diffusion of haemoglobin during the transport of O_2, CO and NO within the red cell (or in haemoglobin solutions of equivalent concentration). The computational work is thereby immensely eased.

These two factors are of such cardinal importance that it will be well to consider in detail, at an early stage, the evidence upon which they are based. It is, in fact, convenient and natural to do so in the next section, dealing with the numerical values of the velocity constants of the haemoglobin reactions and the diffusion coefficients of the dissolved gases, all of which are obviously required for the main purpose of this article.

II. The Velocity Constants of the Reactions of O_2, CO and NO with Haemoglobin

In the preceding chapter on "The Kinetics of Reactions between Haemoglobin and Gases" Professor Q. H. GIBSON has given an extended account of the kinetic theory of the reactions of haemoglobin with O_2, CO and NO in homogeneous solution, together with a description of the methods used for measuring the various velocity constants under different conditions, e.g. of pH, temperature, etc. Actual numerical values for velocity constants of particular haemoglobin reactions will only be given, as and when required in the particular examples in which they are concerned.

The three reactions of haemoglobin, which have been extensively studied *both* in red cell suspensions and in homogeneous haemoglobin solutions prepared from the same blood are:

1. The combination of oxygen with reduced haemoglobin.

* A 30 per cent (w/v) solution contains 30 g of haemoglobin per 100 ml of solution.

2. The combination of carbon monoxide with reduced haemoglobin.

3. The displacement of oxygen from combination with haemoglobin by carbon monoxide, the concentrations of dissolved O_2 and CO being great enough to ensure that no appreciable amount of reduced haemoglobin is present at any stage.

It is only the third of these processes that can be treated throughout its range by a simple kinetic equation (Chapter 1, equation (29)). Provided that the ratio of dissolved O_2 to dissolved CO exceeds 10 to 1, Chapter 1, equation (29) can be further simplified to the following form

$$\frac{d[COHb]}{dt} = \frac{m'[CO][O_2Hb]}{[O_2]} \qquad \ldots.(1)$$

where [COHb], [O_2Hb] represent the total combined [CO] and combined [O_2] in mole/l., and $m' = l'_4 k_4/4k'_4$ (see also Chapter 1, p. 36). In the later stages of the displacement process the velocity of the back reaction becomes increasingly important and it is necessary then to include a second term, $-m$ [COHb], on the right-hand side of equation (1). The complete equation for the whole process, from start to finish, is thus

$$\frac{d[COHb]}{dt} = m'\frac{[CO][O_2Hb]}{[O_2]} - m[COHb] \qquad \ldots.(2.1)$$

or

$$\frac{d[COHb]}{dt} = \mathscr{M}'[CO][O_2Hb] - m[COHb] \qquad \ldots.(2.2)$$

where $\mathscr{M}' = m'/[O_2]$, and is constant provided oxygen is present in such excess as not to change appreciably during the course of the reaction. Equation (2.1) also describes the reverse process, namely the displacement of carbon monoxide from combination with haemoglobin in the presence of excess of O_2. For further discussion of the latter see Chapter 1, p. 38, the symbol l_4 referred to there being equal to our m divided by four.

The kinetics of processes 1 and 2 are severely complicated by the fact that mammalian haemoglobin contains 4 atoms of iron and the combination of haemoglobin either with oxygen or carbon monoxide thus proceeds in four intermediate stages, according to equations (1), (2), (3) and (4) in Chapter 1. Over a considerable range, however, it is found that the rate of process 1 can be approximately described by an empiric bimolecular type of equation

$$\frac{d[O_2Hb]}{dt} = k'[O_2][Hb] \qquad \ldots.(3)$$

where [O_2Hb] represents the total concentration of chemically combined oxygen in mole/l. and [Hb] represents the total concentration of

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uncombined haemoglobin in g-atoms of iron per litre. In buffer solutions containing concentrations of dissolved oxygen ranging from $3{\cdot}6 \times 10^{-5}$ M to $1{\cdot}4 \times 10^{-4}$ M (i.e. solutions in equilibrium with partial pressures of oxygen ranging from about 20 to 75 mm Hg at 19°C), the calculated value of k' for sheep haemoglobin solutions is found only to change by about 20 per cent during the first half of the reaction (see Chapter 1, p. 25 for fuller discussion).

Similarly the early stages of process 2 can be approximately described by the kinetic equation

$$\frac{\mathrm{d}\,[\mathrm{COHb}]}{\mathrm{d}t} = l'\,[\mathrm{CO}][\mathrm{Hb}] \qquad \ldots.(4)$$

the value of l', for $[\mathrm{CO}] = 7 \times 10^{-5}$ M, only increasing by about 25 per cent over the first third of the reaction. Later it will be shown that, in the equations for diffusion plus chemical reaction velocity processes with which we are to deal, the combination velocity constants enter as square root terms, i.e. as $(k')^{1/2}$ or $(l')^{1/2}$, rather than as k' and l', so that a drift in the latter of 25 per cent will only produce an effect of about 12 per cent. By restricting attention, theoretically and experimentally, to sufficiently early phases of the "diffusion plus chemical reaction" processes the uncertainties due to drifts in k' and l' can be cut down to still smaller figures, but—even so—the results cannot be expected to be as clear cut as in process 3, the chemical kinetics of which are, as already mentioned, covered accurately, over the whole course of the reaction, by equation (2.1).

1. *Similarity of the chemical kinetics of the reactions of haemoglobin with gases in the red cell and in dilute solution*

This important matter has already been referred to in the Introduction, wherein it was shown that the rate of uptake of oxygen by sheep haemoglobin in homogeneous solution is about 20 times faster than by the same haemoglobin in the intact red cells (Fig. 1). Obviously the slower the chemical reaction, the less should be the effects of diffusion through the red cell membrane and the red cell interior in cutting down the overall rate of the process in the red cell suspensions. Now the rate of combination of carbon monoxide with sheep haemoglobin in homogeneous solution is much slower than the rate of combination of oxygen, l' in fact being only about one tenth of k'. Corresponding thereto, it is found that in experiments with carbon monoxide, similar to that of Fig. 1 with oxygen, the rate of uptake in homogeneous Hb solution is only two to three times faster than in the red cells, the ratio varying with the particular sheep blood used—as is also the case with the analogous oxygen process.

Finally, in the case of the very slow displacement of carbon monoxide

from combination with sheep haemoglobin by oxygen (i.e. the reverse of process 3 above) the observed rate of the reaction is, within experimental error, *the same* in homogeneous haemoglobin as in the intact red cell, the half-time in both cases being about 60 sec.

TABLE 1

Comparison of Velocity Constants of Three Reactions in Human Haemoglobin Solutions and in Human Red Cell Suspensions at 37°C.

All velocity constants in this table are expressed in units of M^{-1} sec^{-1}.

$O_2 + Hb \rightarrow O_2Hb$	$k' = 1{\cdot}8 \times 10^6$	$k_c' = 9{\cdot}2 \times 10^4$	$k'/k_c' = 19{\cdot}6$
$CO + Hb \rightarrow COHb$	$l' = 6{\cdot}7 \times 10^5$	$l_c' = 7{\cdot}9 \times 10^4$	$l'/l_c' = 8{\cdot}5$
$CO + O_2Hb \rightarrow O_2 + COHb$	$\mathscr{M}' = 1{\cdot}2 \times 10^5$	$\mathscr{M}_c' = 4{\cdot}3 \times 10^4$	$\mathscr{M}'/\mathscr{M}_c' = 2{\cdot}8$
	$\mathscr{M}' = 2{\cdot}1 \times 10^4$	$\mathscr{M}_c' = 1{\cdot}6 \times 10^4$	$\mathscr{M}'/\mathscr{M}_c' = 1{\cdot}3$

Table 1 contains similar data for human haemoglobin solutions and red cell suspensions at 37°C, the actual figures being based on the recent data of GIBSON *et al.* (1955) and of ROUGHTON *et al.* (1957). The symbols k_c', l_c' and $\mathscr{M}_c'$ are new and need definition. During the early stages of oxygen uptake by red cell suspensions the rate of the process can be approximately described by a modification of equation (3) viz.

$$\frac{d\,[\text{average } O_2Hb \text{ in red cells}]}{dt} = k_c'[O_2]\,[\text{average Hb in cells}]$$

or

$$\frac{d\,[O_2Hb]_c}{dt} = k_c'\,[O_2][Hb]_c \qquad \ldots\ldots(5)$$

Similarly for process (2)

$$\frac{d\,[COHb]_c}{dt} = l_c'\,[CO][Hb]_c \qquad \ldots\ldots(6)$$

and for process (3)

$$\frac{d\,[COHb]_c}{dt} = m_c'\frac{[CO][O_2Hb]_c}{[O_2]} - m_c\,[COHb]_c \qquad \ldots\ldots(7.1)$$

$$= \mathscr{M}_c'\,[CO][O_2Hb]_c - m_c\,[COHb]_c \qquad \ldots\ldots(7.2)$$

The values of k_c', l_c' and $\mathscr{M}_c'$ given in Table 1 are the values of these "constants" when extrapolated to zero time. The two different values of $\mathscr{M}_c'$ were obtained by varying $[O_2]$ seven-fold. Just the same trend is again seen as in the case of the sheep material; m and m_c were also found to be equal, within experimental error, in the case of human blood.

The results of these varied experiments seem to the writer to provide good grounds for believing that the differences between the overall

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kinetics of the various haemoglobin reactions in the red cell and in homogeneous solution are, in the main, due to diffusion factors and that the purely chemical kinetics of these reactions may be reasonably assumed to be the same, both qualitatively and numerically, in the two different media. In applying the theoretical "diffusion plus chemical reaction velocity" equations (*vide infra*) to haemoglobin solutions of the concentration found in the interior of the red cell, the actual values for the velocity constants of the various haemoglobin reactions will accordingly be taken to be the same as their respective values in dilute haemoglobin solutions. Should, however, this assumption prove in the future to be wrong, it ought not to be difficult to amend the results of the theoretical calculations, once the correct values for the velocity constants in concentrated solution are definitely known.

III. The Diffusion Coefficients of N_2, O_2, CO and NO in Haemoglobin Solutions of 0–40 Per Cent (w/v)

For gases of low molecular weight, the diffusion coefficients in aqueous solution are inversely proportional to the square root of the molecular weight of the gas. The diffusion coefficients of oxygen, carbon monoxide and nitric oxide in haemoglobin solutions should therefore be calculable, with reasonable accuracy, from the diffusion coefficient of nitrogen, which is easier and more satisfactory to measure in haemoglobin solutions as it engages in no chemical reactions in the latter. Since carbon monoxide has the same molecular weight as nitrogen (and similar solubility), its diffusion coefficient, D_{CO}, should be the same as that of nitrogen, D_{N_2}—this has been confirmed in haemoglobin solution in a number of cases (see later). D_{O_2} should be equal to $(28/32)^{1/2}\ D_{N_2}$, i.e. $0{\cdot}935\ D_{N_2}$, and D_{NO}, which has not—to the writer's knowledge—so far been measured in any haemoglobin solutions, should be equal to $(30/32)^{1/2}\ D_{N_2}$, i.e. $0{\cdot}966\ D_{N_2}$.

1. *Values of* D_{N_2} *in* Hb *solutions*

Longmuir and Roughton (1952) exposed layers of haemoglobin solution of known depth b (usually of the order of 3 cm), to a gas phase containing N_2 at partial pressure p_b for a known time interval t, and measured the nitrogen content of the Hb at the beginning and end of the period. A detailed description of the apparatus and methods of measurement will be found in their paper. Under these circumstances the diffusion of N_2 follows the equation

$$D\frac{\partial^2 c}{\partial x^2} = \frac{\partial c}{\partial t} \qquad \ldots.(8)$$

where x = the distance of any specified cross-section of the Hb solution below the gas–liquid interface,

c = the concentration of dissolved N_2 at x, at time t.

Then if c_b = concentration of dissolved N_2 at the gas–liquid interface where it is constantly in equilibrium with the N_2 in the gas phase, so that $c_b = \alpha p_b$, where α is the solubility coefficient of N_2 in the solution,

c_0 = the initial concentration of dissolved N_2 in the solution,

$\bar{c}$ = the average concentration of dissolved N_2 in the Hb solution at time t (as measured),

it follows (see ROUGHTON, 1932) that, to within 0·2 per cent,

$$\frac{\bar{c} - c_0}{c_b - c_0} \text{ (if less than 0·46)} = \frac{2}{b}\sqrt{\frac{Dt}{\pi}} \qquad \ldots\ldots 9.1)$$

$$\frac{\bar{c} - c_0}{c_b - c_0} \text{ (if greater than 0·46)} = 1 - \exp\left(-\frac{D\pi^2 t}{4b^2}\right) \ldots\ldots (9.2)^*$$

The value of D, the diffusion coefficient of N_2 in the solution, can thus be calculated from the experimental observations with the aid either of equation (9.1) or (9.2), whichever is appropriate.

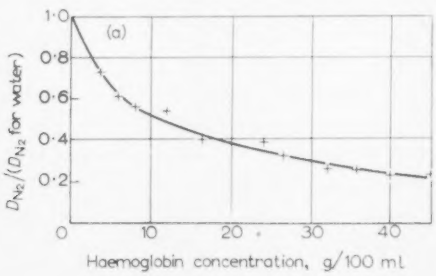

Fig. 2a. Relation between haemoglobin concentration and (diffusion coefficient of N_2 in Hb solution)/(diffusion coefficient of N_2 in water).

Fig. 2*a* shows the relation between (diffusion coefficient of N_2 in Hb solution)/(diffusion coefficient of N_2 in water) plotted vertically and Hb concentration plotted horizontally for haemoglobin solutions prepared from sheep's blood at 20°C. The progressive fall in D_{N_2} as the Hb concentration increases from zero to about 25 per cent (w/v) is explained by the concurrent increase in the viscosity of the solution as is shown by the plateau in Fig. 2*b* in the plot of $D_{N_2} \times \eta$ (viscosity)

*exp (y) = e^y

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against Hb concentration. At Hb concentrations above 30 per cent (w/v) the value of $D_{N_2} \times \eta$ rises sharply due to the fact that the great increase in viscosity in this range is not compensated by anything like a similar decrease in D_{N_2}. The haemoglobin concentration in this range is in fact similar to that found in the interior of mammalian red cells, wherein—as already mentioned—the haemoglobin molecules must be practically touching, and their translatory diffusion coefficient becomes relatively negligible (see later).

Some measurements by the same technique were also made of D_{CO}, the haemoglobin in this case being saturated with CO at a pressure of

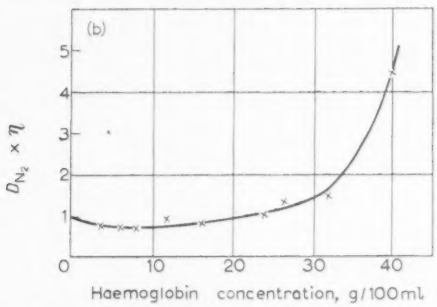

Fig. 2b. Relation between haemoglobin concentration and product of D_{N_2} by viscosity (η).

10 per cent atmosphere previous to exposure of the Hb solution to CO at 1 atm pressure. In this way any concurrent reaction of CO with Hb is eliminated since the Hb is already combined with CO at the start and equations (9.1) and (9.2) can thus be applied as in the case of N_2. Eight comparisons in all were made of D_{CO} and D_{N_2} on the same haemoglobin solutions. The average discrepancy was only 0·25 per cent regarding signs but was 16 per cent disregarding signs. Similar experiments could be made with NO or with O_2, though in the latter case, owing to the lower chemical affinity of Hb for O_2, it would be necessary to saturate the Hb solution initially with O_2 at a pressure of at least 50 per cent atmosphere to insure that there was no significant chemical combination when exposed to an O_2 pressure of 1 atm.

It is assumed that the relationship in Fig. 2*a* also applies to other temperatures besides 20°C and in particular can be used for calculations at mammalian body temperature (37°C), by applying a temperature coefficient of 2·5 per cent/°C. It would be desirable to test this assumption directly, not only for sheep haemoglobin but also for the haemoglobin of other species, especially man.

Example: Given that the value of D_{N_2} in water at 20°C = $2{\cdot}0 \times 10^{-5}$ (cm^2/sec—the usual C.G.S. units in which diffusion

coefficients are expressed), what is the value of D_{O_2} in 30 per cent (w/v) Hb solution?

$$D_{N_2} \text{ in water at } 37°C = (1{\cdot}025)^{(37-20)} \times 2{\cdot}0 \times 10^{-5}$$
$$= 3{\cdot}03 \times 10^{-5}.$$

D_{N_2} in 30 per cent (w/v) Hb solution at 37°C

$$= 3{\cdot}03 \times 10^{-5} \times \frac{D_{N_2} \text{ in 30 per cent (w/v) Hb solution at } 20°C}{D_{N_2} \text{ in water at } 20°C}$$

$$= 3{\cdot}03 \times 10^{-5} \times 0{\cdot}25 \text{ (from Fig. 2}a\text{)}$$

$$= 7{\cdot}6 \times 10^{-6} \text{ cm}^2/\text{sec}.$$

Therefore D_{O_2} in 30 per cent (w/v) Hb solution at 37°C

$$= (\tfrac{2}{3}\tfrac{8}{2})^{\frac{1}{3}} \times 7{\cdot}6 \times 10^{-6}$$
$$= 7{\cdot}1 \times 10^{-6} \text{ cm}^2/\text{sec}.$$

2. *The diffusion coefficient of carbon monoxide and oxygen in haemoglobin solutions by the advancing front method*

The diffusion coefficient of any gas, which combines chemically with haemoglobin, should also be obtainable independently from measurements of the rate at which layers of reduced haemoglobin take up the gas, when exposed to a gas-phase containing that gas (*vide infra*). No exact knowledge is needed of the rate of combination of the gas with haemoglobin, provided that the thickness of the layer of haemoglobin solution is such that the half time of the pure chemical reaction is at least 100 times smaller than the half time of the overall uptake process. This is so in the case of CO, O_2 or NO uptake by haemoglobin layers, provided that the latter are at least 50 μ thick. The results by this method do, in fact, tally reasonably well with those obtained by the previous method 1: such checks are particularly welcome in the case of haemoglobin solutions of the concentration found in mammalian red cells, i.e. 30 per cent (w/v) and upwards, in view of the numerous calculations which have been made on diffusion plus chemical reaction velocity in red cells, wherein a reliable value for the diffusion coefficient for the dissolved gas is obviously a *sine qua non* (Roughton, 1932; Roughton, 1945; Nicolson and Roughton, 1951; Forster *et al.*, 1957; Roughton and Forster, 1957).

The simplest case to start with is that of uptake of carbon monoxide by a layer of 35 per cent (w/v) haemoglobin solution, for in such highly concentrated solutions—as already mentioned—the diffusion of the haemoglobin molecules turns out to be relatively negligible, and it is thus only necessary to take into account the diffusion of the dissolved

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gas. The first CO molecules to diffuse into the layer from the gas above it will combine practically instantaneously with the uppermost haemoglobin molecules so that on the top of the layer there will be a very thin stratum of fully saturated carbon monoxide haemoglobin (COHb), the whole of the haemoglobin beneath being completely reduced. Owing to the great affinity of CO for Hb, the boundary between COHb and Hb will be quite sharply located at a definite "front." As time passes the front steadily advances, as depicted graphically in Fig. 3. The distance

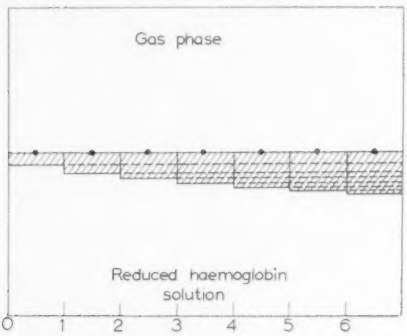

Fig. 3. Picture of advancing front when a layer of reduced haemoglobin solution (in the lower half of the diagram) is exposed to a gas phase containing carbon monoxide (in the upper half of the diagram). The shaded areas represent the depth of the carboxyhaemoglobin zone at a regular series of intervals after the beginning of the exposure.

of the front from the gas/liquid interface, f, can be shown to conform to the equation:

$$f = h_1 (2 D_{CO} c_b t)^{1/2} / [Hb]^{1/2} \quad \ldots . (10)$$

where D_{CO}, c_b and t have the same meanings as in equation (9.1), [Hb] is the concentration of haemoglobin in the layer and h_1 is a factor which depends upon the value of $c_b/[Hb]$, being only significantly different from unity if $c_b/[Hb] > 0{\cdot}2$ (see Table 2).

TABLE 2

Values of the Factor h_1 for Various Values of $c_b/[Hb]$ (Vide *Equations* (10) *and* (11))
The figures in this table are derived from those given in Table V of the paper by A. V. Hill (1929).

$c_b/[Hb]$	0·005	0·0202	0·0824	0·191	0·356	0·592
h_1	1	0·995	0·985	0·97	0·948	0·92
$c_b/[Hb]$	0·92	1·37	2·0	2·86	4·06	8·14
h_1	0·885	0·845	0·8	0·753	0·702	0·59

Since the haemoglobin between the gas/liquid interface and the advancing front is completely saturated with CO, the total CO uptake, V_1, is given by the equation

$$V_1 = f \times [\mathrm{Hb}] = h_1(2D_{\mathrm{CO}}\, c_{\mathrm{b}}\, [\mathrm{Hb}]t)^{1/2} \qquad \ldots.(11)$$

Actually
$$h_1 = g_1 \left(\frac{2[\mathrm{Hb}]}{c_{\mathrm{b}}}\right)^{1/2} \qquad \ldots.(12)$$

where g_1 is the solution of the equation

$$g_1 \,\mathrm{erf}\,(g_1) \exp\,(g_1^2) = \frac{1}{\pi^{1/2}} \frac{c_{\mathrm{b}}}{[\mathrm{Hb}]} \qquad \ldots.(13)$$

and
$$\mathrm{erf}\,(g_1) = \frac{2}{\pi^{1/2}} \int_0^{g_1} \exp\,(-x^2)\,\mathrm{d}x \qquad \ldots.(14)$$

In a special set of experiments LONGMUIR and ROUGHTON (1952) measured simultaneously the rate of diffusion of carbon monoxide *into*

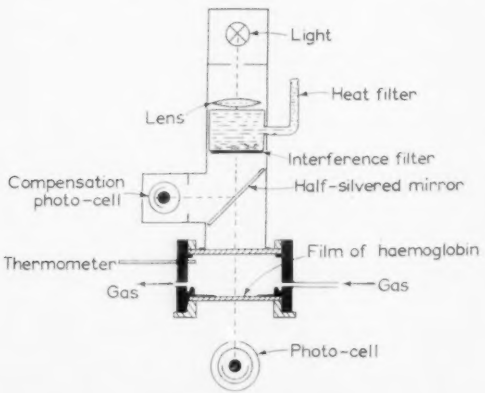

Fig. 4. Schematic diagram of the photo-electronic apparatus used by KREUZER and BETTICHER (1951) for measuring the rate of penetration of oxygen into thin layers of haemoglobin solution. For further particulars, see original paper.

reduced haemoglobin solution and the rate of diffusion of nitrogen *out* of the same haemoglobin solution into the supernatant gas phase, which was initially pure carbon monoxide. The value of D_{CO}, as calculated by equation (10) agreed to within 6 per cent on the average, with the value of $D_{\mathrm{N_2}}$ as calculated by equation (9.1) or (9.2). Such agreement is well within the experimental error of the measurements, and confirms the view that the diffusion of haemoglobin in such concentrated solution may be neglected in comparison with that of carbon monoxide. Actually (see later) the experiments show that D_{Hb}, the diffusion coefficient of haemoglobin, must be less than 0·005 D_{CO} in 35 per cent (w/v) haemoglobin solution.

At haemoglobin concentrations below about 25 per cent (w/v) LONGMUIR and ROUGHTON found that D_{CO} tended to be increasingly greater than D_{N_2} which implied that the effect of diffusion of the haemoglobin molecules was no longer negligible. A similar state of affairs is revealed by a study of the results obtained in the Department of Physiology of the University of Fribourg (MÜLLER, 1945, 1948; LASZT, 1945; PIRCHER, 1951, 1952; KREUZER, 1950, 1951, 1953) on the rate of penetration of oxygen into very thin layers of haemoglobin

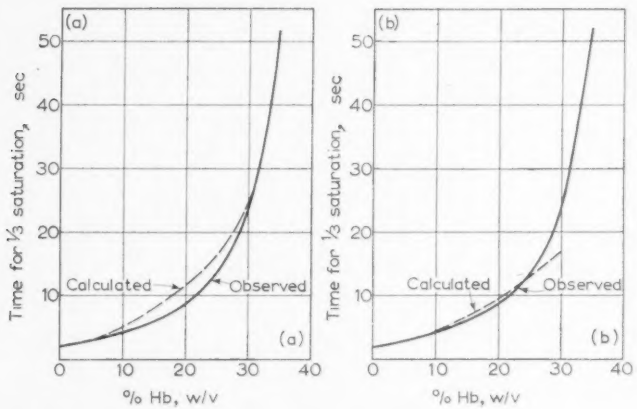

Fig. 5a. Full curve gives relation between time for one third saturation (as observed experimentally) and percentage Hb (w/v), for a film 100μ thick when exposed to oxygen at 0·9 atmosphere at 25°C. The broken line curve gives the relation, as calculated by equation (10), the diffusion of haemoglobin being neglected.

Fig. 5b. Full line curve represents the same observed results as in Fig. 5a. The broken line curve gives the relation, as calculated by equation (15), which allows for the diffusion of haemoglobin. It is assumed that $D_{Hb} = 0{\cdot}03\ D_{O_2}$.

solution (50 μ upwards in thickness). The elegant apparatus (see Fig. 4) and photo-electronic technique by which the course of the process was followed are described by KREUZER (1950) and KREUZER and BETTICHER (1951). In Fig. 5*a*, the full line curve shows the relation between Hb concentration (horizontal) and the observed times for one-third saturation of films 100 μ thick, when exposed to oxygen at 0·9 atm at 25°C. The broken line, on the other hand, gives the calculated times for one-third saturation on the advancing front theory, but assuming that the haemoglobin is immobile and the values of D_{O_2} at each Hb concentration can be obtained from Fig. 2*a* of the present paper as described above. The area between the full line curve and the broken line curve in Fig. 5*a* displays graphically the effect of the haemoglobin diffusion at lower concentrations. This method of portrayal was given in Fig. 2 of a paper by KLUG *et al.* (1956), but a

rechecking of their calculations has shown that the effect of haemoglobin diffusion was markedly less than that given in their Fig. 2.

When diffusion of haemoglobin also has to be taken into account equation (11) must be replaced by the following:

$$V_2 = h_2(2D_{CO}\, c_b\, [Hb]t)^{1/2} \qquad \ldots.(15)$$

where

$$h_2 = \frac{2\exp(-g_2^2)}{\pi^{1/2}\,\mathrm{erf}\,(g_2)}\left(\frac{c_b}{2[Hb]}\right)^{1/2} \qquad \ldots.(16)$$

and g_2 is the solution of the equation

$$\frac{c_b}{[Hb]}\left(\frac{D_{CO}}{D_{Hb}}\right)^{1/2}\exp\left(g_2^2\,\frac{D_{CO}-D_{Hb}}{D_{Hb}}\right) = \frac{\mathrm{erf}\,(g_2)}{1-\mathrm{erf}\left(g_2\left[\frac{D_{CO}}{D_{Hb}}\right]^{1/2}\right)} \qquad \ldots.(17)$$

Equation (17) can be solved numerically for g_2 for any pair of values of $[Hb]/c_b$ and D_{CO}/D_{Hb}, and h_2 is then calculated from g_2 by equation (16). Table 3 gives the calculated values of h_2 for the range of values of

TABLE 3

Values of h_2 for various Values of $[Hb]/c_b$ and D_{Hb}/D

		$\frac{[Hb]}{c_b}$							
		1	5	10	20	30	50	70	100
$\frac{D_{Hb}}{D}$	0·000	0·88	0·97	0·98	0·99	1·0	1·0	1·0	1·0
	0·010			1·035	1·08	1·113	1·193	1·25	1·385
	0·020	0·92	1·01	1·07	1·16	1·22	1·33	1·44	1·58
	0·030		1.04	1·105	1·23	1·31	1·43	1·58	1·77

$[Hb]/c_b$ and D_{CO}/D_{Hb}, which are likely to be met with in experimental studies of the rate of penetration of CO or other gases, into layers of haemoglobin solution. Exactly the same figures apply, of course, to the penetration of NO (with D_{NO} substituted for D_{CO}) and to the penetration of O_2 (with D_{O_2} substituted for D_{CO}), though in the latter case, owing to the much lower affinity of oxygen for haemoglobin, there will be some "blurring" or "no man's land" at the line of the advancing front and the theoretical analysis will not be quite so satisfactory.

Polson (1939) measured the value of D_{Hb} over the range 0–4 per cent (w/v) haemoglobin solution, but owing to the depth of colour of haemoglobin solutions he could not, with the optical method at his disposal, extend his results to higher concentrations. In very dilute

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solution he found that $D_{Hb} = 0{\cdot}03\ D_{O_2}$: the broken line curve in Fig. 5*b* represents the calculated results with values of D_{O_2} as in Fig. 5*a* but with D_{Hb} assumed to be $0{\cdot}03\ D_{O_2}$ at every haemoglobin concentration. Between 0 and 25 per cent (w/v) Hb, the calculated curve lies somewhat above the observed curve, but the trend—though apparently definite—is not outside the limits of experimental error. Above 25 per cent Hb (w/v) there is an unmistakable discrepancy due to the sharp fall in D_{Hb} in this range (in association with the corresponding rise in viscosity), the value of D_{N_2} (see Fig. 2*a*)—and inferentially that of D_{O_2}—only falling far more gradually, so that D_{Hb}/D_{O_2} becomes much less than 0·03.

Earlier in this section it has been pointed out that LONGMUIR and ROUGHTON's simultaneous measurements of D_{N_2} and D_{CO}, by the advancing front method, gave an upper limit to the value of D_{Hb}/D_{CO} in 35 per cent (w/v) Hb solutions. Since their results showed that D_{CO} was not more than 1·06 times D_{N_2}, the value of h_2 could not have exceeded $(1{\cdot}06)^{1/2}$, i.e. 1·03. For a 35 per cent (w/v) Hb solution exposed to CO at 1 atm pressure the value of $[Hb]/c_b$ is about 20, and by interpolation from the figures in Table 3 it can be seen that the value of D_{Hb}/D_{CO}, corresponding to $h_2 = 1{\cdot}03$ and $[Hb]/c_b = 20$, is 0·005. From this it follows that D_{Hb} in 35 per cent (w/v) Hb solution $\gtrsim 0{\cdot}005 \times D_{CO}$ in 35 per cent Hb $\gtrsim 0{\cdot}005 \times 4 \times 10^{-6}$ cm²/sec $\gtrsim 2 \times 10^{-8}$ cm²/sec, i.e. about 0·03 of the value of D_{Hb} in dilute solution which, according to POLSON (1939), is about $6{\cdot}9 \times 10^{-7}$ cm²/sec at 20°C. It would clearly be of interest to obtain direct values of D_{Hb} in the range 3–40 per cent (w/v) Hb solution, but the writer has not found any such data in the literature. It would seem feasible to obtain them from measurements of the rate of diffusion of "tagged" haemoglobin into untagged haemoglobin solutions, the haemoglobin being tagged e.g. with CO, NO or radioactive iron. More precise work on the diffusion of gases in haemoglobin solutions, in which the diffusion of haemoglobin is also a limiting factor, should be deferred until (or unless) values of D_{Hb} over the whole concentration range are available. It would also theoretically be preferable to study the diffusion of CO or NO rather than that of O_2, in view of the possibility of "blurring" of the advancing front in the latter case, as mentioned above.

Equations (11), (12), (13), (15), (16) and (17) are all particular cases of more general equations met with in analogous problems in the theory of the conduction of heat (e.g. in progressive melting or freezing), which go back at least as far as the work of STEFAN (1890) and are developed and reviewed in a very lucid way by CARSLAW and JAEGER (1947, §§ 31 and 107); see also DANCKWERTZ (1950) and CRANK (1956, 1957).

Advancing front equations were also obtained independently by

A. V. HILL (1929) in his classical paper on *The diffusion of oxygen and lactic acid through tissues*, wherein the reader will find many interesting pioneer applications of such equations to problems of diffusion in muscle and in nerve.

PIRCHER (1951, 1952) and recently THEWS (1957) have interpreted in an alternative way the Fribourg data on the rate of penetration of oxygen into layers of haemoglobin solution. Unfortunately, however, their methods are—in the opinion of the writer—open to serious criticism, some of which is based on work described later in the present review. It will therefore be convenient to defer discussion of this work to a subsequent section (see pp. 82 to 86).

IV. DIFFUSION PLUS CHEMICAL REACTION IN HAEMOGLOBIN SOLUTION (WITHOUT MEMBRANES)

So far chemical reaction velocity constants have not appeared in any of the diffusion equations of this article, the reasons being either, as in the case of nitrogen, that no chemical reactions have been involved or, as in the case of the advancing front problems, that the distance over which diffusion occurs is such that the total time of the whole gas uptake process is enormously greater than the time of the purely chemical reaction. In the latter circumstances it has been legitimate to treat the chemical reaction as "instantaneous."

We have now to consider the diffusion of gases into much thinner haemoglobin layers, of thickness down to 1–2 μ, wherein the tempo of the overall process is no longer of a different order from that of the chemical reactions of haemoglobin with such gases. "Simultaneous diffusion plus chemical reaction velocity" processes of this kind have been much studied in recent years (see e.g. CRANK, 1956 for a valuable presentation of the subject; also DANCKWERTZ, 1950, 1951; PERRY and PIGFORD, 1953; O'SULLIVAN, 1955). Although the general methods described in these works are indispensable for a start on the haemoglobin problems, it has been necessary, in pursuing them more fully, to introduce a number of special lines of attack, as has been done over the past 25 years by ROUGHTON and his colleagues (ROUGHTON, 1932; ROUGHTON, 1945, NICOLSON and ROUGHTON, 1951; ROUGHTON, 1952; KLUG *et al.*, 1956).

1. *Diffusion plus chemical reaction in thin membraneless layers of haemoglobin solution*

Penetration of O_2 (*or* CO) *into reduced* Hb *layers.* Assuming that the concentration of haemoglobin in the layer is greater than 35 per cent (w/v) so that the diffusion of haemoglobin can be neglected, the equations governing the process of oxygen penetration into a layer of

reduced haemoglobin of thickness $2b$ and exposed on both sides to a constant pressure of the gas, p_b (as depicted in Fig. 6) are as follows:

$$D\frac{\partial^2 c}{\partial x^2} = \frac{\partial c}{\partial t} + k'c\,[\mathrm{Hb}] - k\,[\mathrm{O_2Hb}] \qquad \ldots.(18)$$

and

$$\frac{\mathrm{d}[\mathrm{O_2Hb}]}{\mathrm{d}t} = k'c\,[\mathrm{Hb}] - k\,[\mathrm{O_2Hb}] \qquad \ldots.(19)$$

subject to the boundary conditions:

1. At $t = 0$, $c = 0$ for all values of x within the layer.
2. At $x = b$, $c = c_b = \alpha\, p_b$ at all times.
3. At $x = 0$, the centre of the layer, $\dfrac{\partial c}{\partial x} = 0$ at all times.

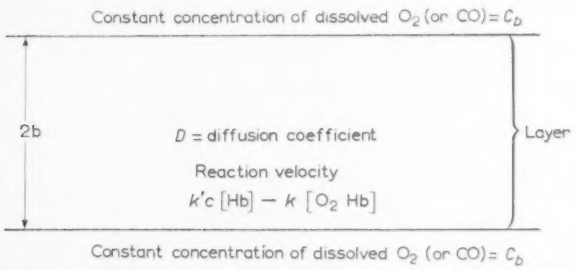
Constant concentration of dissolved O2 (or CO) = Cb
2b
D = diffusion coefficient
Reaction velocity
k'c [Hb] − k [O2 Hb]
Layer
Constant concentration of dissolved O2 (or CO) = Cb

Fig. 6. Scheme of diffusion of dissolved gas into a membraneless layer of haemoglobin solution. Cf. Fig. 17.

These equations are not in general capable of formal analytical solution. In the earliest stages of the process, however, [Hb] may be taken as approximately constant at its initial value $[\mathrm{Hb}]_0$ and $[\mathrm{O_2Hb}]$ may be neglected. Replacing the expression $k'\,[\mathrm{Hb}]_0$ by a single constant κ' we then have

$$D\frac{\partial^2 c}{\partial x^2} = \frac{\partial c}{\partial t} + \kappa' c \qquad \ldots.(20)$$

The analytical solution of equation (20), subject to the above boundary conditions is

$$c = c_b\left[\frac{\cosh\left(x\sqrt{\frac{\kappa'}{D}}\right)}{\cosh\left(b\sqrt{\frac{\kappa'}{D}}\right)} - \frac{2}{b}\sum_0^\infty \frac{(-1)^n \dfrac{2n+1}{2b}\pi}{\left(\dfrac{2n+1}{2b}\pi\right)^2 + \dfrac{\kappa'}{D}} \times \right.$$

$$\left. \cos\left(\frac{2n+1}{2b}\pi x\right) \exp\left\{-\left(\frac{2n+1}{2b}\pi\right)^2 Dt - \kappa' t\right\}\right] \qquad \ldots.(21)$$

The average concentration of O_2 in physical solution in the layer at time t (ROUGHTON, 1932, equation VIII)

$$= \frac{1}{b}\int_0^b c \, dx \qquad \ldots.(22)$$

$$= \frac{c_b}{b}\left[\sqrt{\frac{D}{\kappa'}}\tanh\left(b\sqrt{\frac{\kappa'}{D}}\right) - \frac{2}{b}\sum_0^\infty \frac{\exp\left\{-\left(\frac{2n+1}{2b}\pi\right)^2 Dt - \kappa' t\right\}}{\left(\frac{2n+1}{2b}\pi\right)^2 + \frac{\kappa'}{D}}\right] \qquad \ldots.(23)$$

whereas the average concentration of combined O_2, i.e. $[\overline{O_2Hb}]$, at time t

$$= \frac{\kappa'}{b}\int_0^t\int_0^b c \, dt \, dx \qquad \ldots.(24)$$

$$= \frac{\kappa' c_b}{b}\left[t\sqrt{\frac{D}{\kappa'}}\tanh\left(b\sqrt{\frac{\kappa'}{D}}\right) - \frac{2D}{b}\sum_0^\infty \frac{1 - \exp\left\{-\left(\frac{2n+1}{2b}\pi\right)^2 Dt - \kappa' t\right\}}{\left\{\left(\frac{2n+1}{2b}\pi\right)^2 D + \kappa'\right\}^2}\right] \qquad \ldots.(25)$$

Equations (21), (23) and (25) at first sight appear rather formidable but their bark is worse than their bite, since it is found that the infinite series on the right hand side of equations (21), (23) and (25) is in each case what is called a "transient" expression, the sum of its terms soon fading out into a negligible quantity as t increases. Of most importance for the present purpose is equation (25). Since $\tanh x = 1$ to within 1 per cent, if x is greater than 2·7, equation (18) after the disappearance of the initial transient series simply becomes

$$[\overline{O_2Hb}] = \frac{c_b t\sqrt{D\kappa'}}{b} \qquad \ldots.(26)$$

It is instructive to note that in this case of diffusion plus chemical reaction velocity the time for a given amount of total oxygen uptake is proportional to the actual thickness of the layer and not to the square of the thickness, as in a process solely of diffusion without chemical reaction (see equations (9.1) and (9.2)). In a purely chemical kinetic process, on the other hand, the time for a given amount of change is

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independent of the linear dimension, b, so that equation (26) for diffusion plus chemical reaction velocity is a kind of geometrical mean between pure diffusion and pure chemical reaction.

The treatment so far given is only valid for a very short time after the start of the process, in view of the progressive decline in [Hb] from its initial value $[\overline{\mathrm{Hb}}]_0$ and the parallel rise in $[\mathrm{O_2Hb}]$. ROUGHTON (1932) succeeded in coping to some extent with this difficulty by two sets of

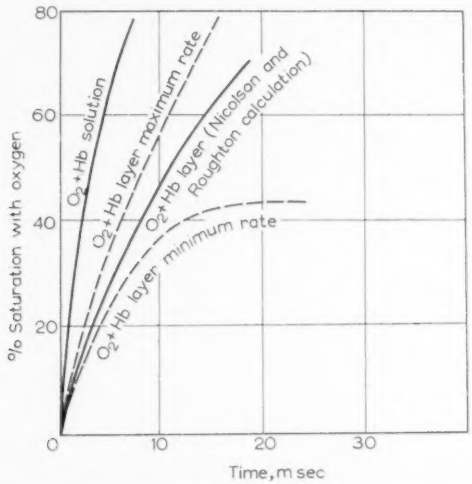

Fig. 7. Comparison of rate of uptake of oxygen by haemoglobin in homogeneous solution with (i) maximum calculated rate of uptake by a layer of haemoglobin solution of concentration and thickness corresponding to the values found in the red cell, (ii) minimum calculated rate of uptake of oxygen by the same layer and (iii) rate of uptake of oxygen by the layer, as calculated by method of NICOLSON and ROUGHTON (1951).

simplifying assumptions, one of which led to a mathematical solution giving a value of $[\overline{\mathrm{O_2Hb}}]$ which must always be greater than the true value, whereas the second set of assumptions led to a *minimal* solution for $[\mathrm{O_2\overline{Hb}}]$. Fortunately it was found that the maximal and minimal solutions did not diverge from each other by more than 1 part in 5 over the first third of the overall oxygen uptake process (see Fig. 7). The arithmetical mean of these two extreme solutions must therefore have been correct to within 10 per cent or better.

The penetration of carbon monoxide into very thin layers of concentrated haemoglobin solution follows exactly the same equations as for oxygen, except of course that l' replaces k' and the back-reaction term $l\,[\mathrm{COHb}]$ is to all intents and purposes negligible until much later in the overall process. The maximal and minimal solutions in this case only differed by about 1 part in 8, so that their arithmetic mean is

certainly within 6 per cent of the true solution and is probably much closer (see Fig. 8).

Twenty years after ROUGHTON's first work on this subject had been published NICOLSON and ROUGHTON (1951) took up these problems again and applied to them modern methods of computation based upon the calculus of finite differences. The detailed numerical methods used in their paper do not lend themselves to brief exposition, and there seems no alternative but to refer the reader, who wishes full information thereon, to the original source. An immediate—and it must be

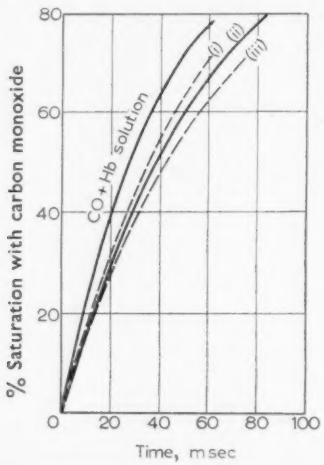

Fig. 8. Similar comparison to those of Fig. 7 but for carbon monoxide. Curves labelled (i), (ii) and (iii) correspond to similar curves in Fig. 7.

admitted—a very gratifying finding was that the course of oxygen and carbon monoxide uptakes by the haemoglobin layers, as calculated numerically by NICOLSON and ROUGHTON (1951), agreed to a singular degree of exactitude with the arithmetic mean of the maximal and minimal solutions given by ROUGHTON in 1932 (see Figs. 7 and 8). It is indeed remarkable how often, in this general field of work, rather crude methods of the latter type have given results in close accordance with those subsequently obtained by highly respectable methods of calculation.

The actual numerical results for the rates of oxygen and carbon monoxide uptake by haemoglobin layers of similar concentration and thickness to those of the mammalian red cell were always found, both in the case of sheep blood (see Fig. 1) and in the case of human blood, to be considerably faster than the experimentally observed rates of uptake in these gases by red cell suspensions. The discrepancy, as already mentioned in the Introduction, is attributable to the resistance

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of the red cell membrane to the passage of the dissolved gases. NICOLSON and ROUGHTON (1951) accordingly extended their theoretical computations to layers of haemoglobin solution enclosed in membranes of varying permeability; before discussing this work and more recent developments thereof it will first be desirable to review the work of KLUG *et al.* (1956) on the rate of displacement of oxygen from combination with haemoglobin by carbon monoxide in layers of solution of 50 μ thickness and upwards, for in this case it has proved feasible to check the theoretical formulae of the present Section directly against actual experimental observations on thin layers of haemoglobin solution.

1.1. *Displacement of* O_2 *by* CO *in* O_2Hb *layers of* 50 *to* 100 μ *thickness.* For really satisfactory tests of the applicability of the methods of NICOLSON and ROUGHTON (1951) to diffusion plus chemical reaction in layers of Hb of thickness $\geq 50\ \mu$ (i.e. the thinnest layers accessible to direct study *in vitro* by the photo-electronic methods of the Fribourg school), it is necessary to find a gas–Hb reaction with a true chemical rate of such a size that the overall observed rate in films of this thickness would be about one-half to one-quarter of that in homogeneous solution. A reaction which well meets this requirement is the displacement of O_2 from combination with Hb, when layers of O_2Hb are exposed to gas mixtures consisting of 1 per cent CO in air or 1 per cent CO in oxygen. At this ratio of CO to O_2, the true chemical kinetics of this displacement reaction conform to equations (2.1) and (2.2) over the whole range of the reaction, whereas in the cases of the reactions $O_2 + Hb \rightarrow O_2Hb$ and $CO + Hb \rightarrow COHb$ which have already been discussed the relevant kinetic equations, viz. (3) and (4) only hold good over a limited initial range of the homogeneous chemical reactions. The displacement reaction is therefore theoretically, as well as experimentally, much better suited to our present purpose. Actual measurements of the rate of the displacement reaction were made with the apparatus of KREUZER and BETTICHER (1951), O_2Hb solutions of concentrations ranging from 3 to 8 per cent (w/v) being exposed in layers of 50 or 100 μ thickness to 1 per cent CO in air or to 1 per cent CO in O_2.

To begin with we shall neglect the diffusion of the haemoglobin molecules although, as shown later, this factor is of appreciable though not great significance in the present range of haemoglobin concentration (3–8 per cent (w/v)). The governing equations, with allowance for the back reaction but not for haemoglobin diffusion, are as follows:

$$D' \frac{\partial^2 c'}{\partial x^2} = \frac{\partial c'}{\partial t} + \mathscr{M}' c' [O_2Hb] - m [COHb] \qquad \ldots.(27)$$

$$\frac{d [COHb]}{dt} = \mathscr{M}' c' [O_2Hb] - m [COHb] \qquad \ldots.(28)$$

with the boundary conditions

$$\left.\begin{aligned} c' &= 0 \\ [O_2Hb] &= [O_2Hb]_0 \end{aligned}\right\} \text{for } 0 \leq x < b \text{ at } t = 0$$

$$c' = c'_b = \alpha' p'_b \text{ at } x = b \text{ for all times}$$

$$\frac{\partial c'}{\partial x} = 0 \text{ at } x = 0 \text{ for all times}$$

Symbols with primes in the case of CO correspond to symbols without primes in the case of O_2. $[O_2Hb]_0$ is the initial concentration of oxyhaemoglobin at zero time.

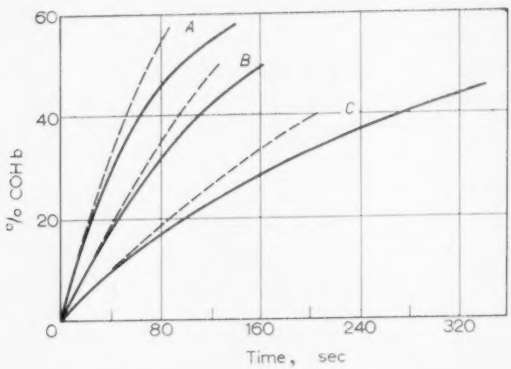

Fig. 9. Calculated rates of displacement of oxygen from combination with haemoglobin when exposed to gas mixtures containing 1 per cent CO + 99 per cent O_2. In the broken line curves no allowance is made for the back reaction: in the full line curves the back reaction is taken into account. The cases treated are: (A) The pure chemical reaction with $m' = 1{\cdot}33$, $m = 0{\cdot}005$; (B) A 3 per cent (w/v) Hb solution in a layer 50μ thick with the same values of m' and m, $D_{CO} = 1{\cdot}2 \times 10^{-5}$ cm^2/sec; (C) A 3 per cent (w/v) Hb solution in a layer 100μ thick.

The paper of KLUG *et al.* (1956) should be consulted for the details of the numerical computation which was generally similar to that of NICOLSON and ROUGHTON (1951). A typical example of the work of KLUG *et al.* (1956) is given in Fig. 9, which shows the effect of varying the thickness of the layer from (B) 50 μ to (C) 100 μ in the case of a 3 per cent (w/v) solution of Hb exposed to 1 per cent CO + 99 per cent O_2. For comparison the rate of the pure chemical reaction (A) is also included. The values for the velocity constants in this example were $m' = 1{\cdot}33$ and $m = 0{\cdot}005$.

The broken line curves represent the calculated results without allowing for the back reaction term, m [COHb], whereas in the full line curves the back reaction is properly taken into account. In the case of

the pure chemical reaction (A) and the thinner layer (B), the back reaction does not exert an appreciable influence until 20 per cent COHb is reached. In the thicker layer (C) the back reaction makes itself felt at an earlier stage; this is physico-chemically reasonable, since with the slower tempo in the latter case the haemoglobin in the layer will have more time in which to approach the final equilibrium distribution between combination with CO and with O_2. In all three cases, however, the back reaction is seen to decrease the calculated rate (as given by the inverse of the time taken to reach a given saturation) by roughly the same amount, i.e. 15–20 per cent.

We must now consider the effect of haemoglobin diffusion—a factor which was quite legitimately neglected in the earlier work of NICOLSON and ROUGHTON (1951), since their computations were limited to layers of concentrated haemoglobin solution. In the case of the more dilute solutions used in the work of KLUG *et al.* (1956) the relevant equations, if the back reaction velocity term be neglected, are

$$\frac{D'\,\partial^2 c'}{\partial x^2} = \frac{\partial c'}{\partial t} + \mathscr{M}' c'\,[\mathrm{O_2Hb}] \qquad \ldots.(29)$$

$$D_{\mathrm{Hb}}\frac{\partial^2 [\mathrm{O_2Hb}]}{\partial x^2} = \frac{\partial [\mathrm{O_2Hb}]}{\partial t} + \mathscr{M}' c' [\mathrm{O_2Hb}] \qquad \ldots.(30)$$

Equations (29) and (30) take into account the diffusion of carbon monoxide and of haemoglobin but not that of oxygen. In the liquid phase c' (i.e. CO concentration) varies of course with time and distance from the gas–liquid interface, but c (i.e. O_2 concentration) is essentially constant since it can be shown by a straightforward calculation, such as is given in ROUGHTON's (1932) paper, that any excess of dissolved O_2 would, in a typical layer of 50 μ, be reduced to half its value in only $\frac{1}{2}$ sec, a time negligible in comparison with those that arise in the actual experiments. It is accordingly legitimate to omit any corrections for limiting effects due to oxygen diffusion under these circumstances.

The numerical handling of equations (29) and (30) is fully set out in Table 1 of the paper of KLUG *et al.* (1956a); it was based on the method which had been used successfully in problems of heat conduction when there is a simultaneous chemical reaction (CRANK and NICOLSON, 1947) or phase change (KLUG, 1953).

An example of the calculated effect of haemoglobin diffusion is shown in Fig. 10 for the case of a 50 μ layer of 6 per cent (w/v) Hb solution exposed to 0·6 per cent CO + 99·4 per cent, O_2, $m' = 5{\cdot}33$. The back reaction is neglected. In curve A, $D_{\mathrm{Hb}} = 0$ whereas in curve B, $D_{\mathrm{Hb}} = 3{\cdot}1 \times 10^{-7}$ cm^2/sec, i.e. one-fortieth of the value of D' (for CO). The diffusion of haemoglobin is seen to *increase* the calculated

rate by about one-sixth, i.e. by about the same amount as the back-reaction in Fig. 9 decreased the calculated rate.

No computations were made by KLUG *et al.* (1956a) for the complete case, in which both the back reaction and haemoglobin diffusion are allowed for, but in the present type of work it is frequently found that such small effects are approximately additive, and this will be assumed to be the case in the comparisons with actual experiment which are now to be described.

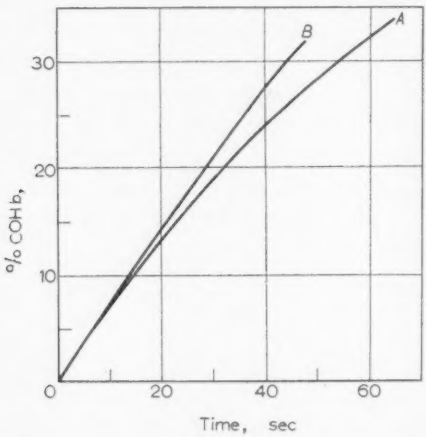

Fig. 10. Effect of haemoglobin diffusion on rate of displacement of oxygen from combination with haemoglobin in layer of 6 per cent Hb (w/v) solution 50μ thick: Curve A—Diffusion of haemoglobin neglected; Curve B—$D_{Hb} = 3{\cdot}1 \times 10^{-7}$ cm²/sec. For further detail see text.

Fig. 11 shows graphically the results of the most complete comparison so far made. In this case a 100 μ layer of 3 per cent Hb solution was exposed to 1 per cent CO + 99 per cent O_2 for a period of 15 min at 18·5°C, the final end-point being reached at about 66 per cent COHb. The circles and the smooth line, labelled E, drawn through them represent the observed points up to 10 min from the beginning of the process. Curve S, which is included for the sake of comparison, shows the rate of the pure chemical reaction in homogeneous solution at the same reagent concentrations and temperature as in curve E. In the calculations, the independently determined values of $m' = 1{\cdot}2$ and $m = 0{\cdot}0045$ were used and D_{CO} was taken to be $1{\cdot}5 \times 10^{-5}$ cm²/sec. The broken curve A, covering the first two-thirds of the whole process, is calculated on the assumption that the diffusion of Hb and the back reaction are both so slow that they can be neglected. If the back reaction is included, but the diffusion of Hb is still neglected, the broken curve B is obtained. If curve B is corrected for Hb diffusion by the additivity principle, curve C finally results. The latter curve is seen to

agree very closely (i.e. to within about 5 per cent) with the experimental curve E, over two-thirds of its course. The calculated curves were not continued beyond this range, owing to the complexity and uncertainty of the computations when the process is getting near its end-point.

In a total of 14 experiments on blood from two different sheep at two Hb concentrations (3 per cent and 6 per cent (w/v)), two layer thicknesses (50 and 100 μ) and two different values of $\mathscr{M}'$ the calculated times for one-third or one-half of the total displacement process

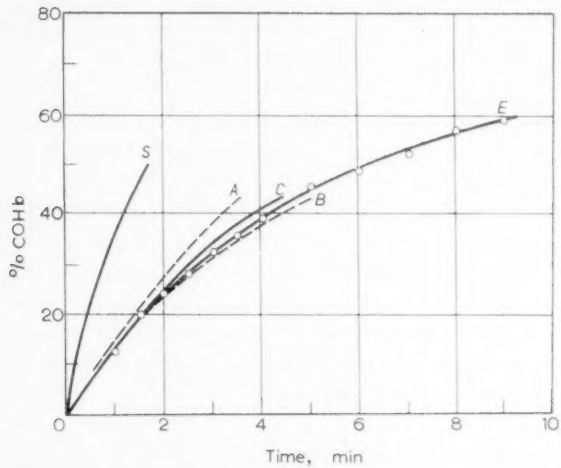

Fig. 11. Comparison of an observed and calculated reaction curve for a 100μ layer of 3 per cent Hb (w/v), other conditions as stated in text: (A) Calculated curve neglecting back-reaction and Hb diffusion; (B) calculated curve taking into account back-reaction but neglecting Hb diffusion; (C) calculated curve taking into account both back-reaction and Hb diffusion; (E) experimental curve; (S) rate of true chemical reaction in homogeneous solution.

agreed to within 8 per cent on the average with the corresponding observed times. Considering that the calculated times depend on the experimental values of m', m, D' and D_{Hb}, none of which is known to an accuracy of better than 5–10 per cent, such an agreement is certainly as good as could have been expected, and does indeed provide a strong confirmation of the whole theoretical basis of the treatment of diffusion plus chemical reaction velocity in this article and in similar writings by other authors.

Besides the particular examples quoted above, KLUG *et al.* (1956a) also give "master" plots (i) of the time to reach various percentages saturation as a function of the parameter $q' = [\mathrm{Hb}]\mathscr{M}'b^2/D'$, and (ii) of the influence of D_{Hb} on the value of q'. For further information on these and other more general matters the reader is referred to the original papers.

1.2. *A criticism of Thews' interpretation of experiments on the rate of penetration of oxygen into thin layers of haemoglobin solution.* In a recent paper THEWS (1957) has given a very ingenious method for calculating the diffusion coefficient of oxygen in haemoglobin solutions from data, such as those provided by KREUZER (1950) and his colleagues in their studies on the rate of penetration of oxygen into thin layers of haemoglobin solution. Previously PIRCHER (1951, 1952) had applied a much more limited method of a similar type to the Fribourg data but since the same criticisms apply to his work as to the more general studies of THEWS, comments will for reasons of space be limited to the recent paper of THEWS, which is based on the following principle.

If a diffusing substance is immobilized by reacting chemically with some non-diffusing substance, which is distributed throughout the medium in which diffusion is occurring, and if furthermore the chemical reaction proceeds very rapidly compared with the diffusion process, it can be assumed that local equilibrium exists everywhere in the medium between the free and the chemically combined components of the diffusing substance. The simplest case to consider—and indeed the only one which can be easily handled mathematically—is that in which the concentration of the combined substance, y, is directly proportional to its concentration in free solutions, i.e.

$$y = Rc \qquad \ldots.(31)$$

where R is a simple partition equilibrium constant. Equation (8) of the present review is then replaceable by the equation

$$\frac{D}{R+1}\frac{\partial^2 c}{\partial x^2} = \frac{\partial c}{\partial t} \qquad \ldots.(32)$$

or

$$D''\frac{\partial^2 c}{\partial x^2} = \frac{\partial c}{\partial t} \qquad \ldots.(33)$$

where

$$D'' = D/(R+1) \qquad \ldots.(34)$$

The progress of the overall diffusion will then conform to equations (9.1) and (9.2) but with D'' substituted for D therein. If D'' is determined in this way and if furthermore R is independently known or deduced, the value of D is readily calculated from equation (34). For further discussion of equations (32), (33) and (34) the reader is referred to Chapter VIII of CRANK's (1956) book.

In applying this principle to the diffusion of oxygen into haemoglobin solutions THEWS was faced with the difficulties (i) that the relation between y and c is not linear but sigmoid, (ii) that at the oxygen pressures in which he was primarily interested, namely 140 mm Hg

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and 700 mm Hg, the haemoglobin in the neighbourhood of the gas–liquid interface will become saturated with oxygen very rapidly and the corresponding value of R will drift very rapidly down to zero. The value of R thus depends both on x and t, but THEWS considered that his mathematical procedure enabled him to obtain a sufficiently satisfactory *average* value of R during the diffusion process, for use in equations (32), (33) and (34).

The two main objections are as follows:

(α) It is tacitly assumed that the diffusion of haemoglobin is negligible over the whole concentration range used in the Fribourg studies. As already indicated this assumption is only sound in very dilute solution and in solutions of 30 per cent Hb (w/v) and higher. At moderate haemoglobin concentrations in the range of 15–20 per cent (w/v) diffusion of haemoglobin will introduce an effect of the order of $([\mathrm{Hb}] \times D_{\mathrm{Hb}})/([\mathrm{O}_2] \times D_{\mathrm{O}_2})$: in 15 per cent (w/v) Hb solution at an oxygen pressure of 740 mm Hg this fraction is equal to about 0·27 (assuming that $D_{\mathrm{Hb}} = 0{\cdot}03 D_{\mathrm{O}_2}$), whilst in 15 per cent (w/v) solution at an oxygen pressure of 140 mm Hg the fraction is about 1·35, so that diffusion of haemoglobin might in this case be of equal or even greater importance than that of oxygen.

(β) It is impossible, through lack of space, to give a full description of THEWS' mathematical procedure, for which the original paper must be consulted. The present writer does not, however, consider that THEWS has established the mathematical justification of his method of averaging and feels that agreement in the values of D_{O_2} by his method with those by other methods may be fortuitous. This belief is supported by experiences gained in comparing the results of calculations by THEWS' method with those by the advancing front method and by the method of NICOLSON and ROUGHTON (1951). In order to avoid uncertainties due to haemoglobin diffusion the calculations have been made on 30 per cent (w/v) haemoglobin solutions: in an accompanying paper on oxygen diffusion in the lung THEWS (1957) has applied his method to haemoglobin layers of the same order of thickness as that of the red cell, and the writer has also—in the examples now to be quoted—chosen layers of this thickness, since they have the advantage that the method of NICOLSON and ROUGHTON is also readily applicable to them.

Fig. 12 gives the results of a comparison of THEWS' method and the method of the advancing front. The calculations are for a layer of 30 per cent (w/v) Hb solution, 7×10^{-5} cm thick, exposed to an oxygen pressure of 700 mm Hg at 25°C, the value of D_{O_2} being assumed to be 5×10^{-6} cm²/sec. The time taken to reach 50 per cent saturation, $t_{\frac{1}{2}}$, turns out to be the same, so that if D_{O_2} had been calculated by THEWS' method from $t_{\frac{1}{2}}$ the value obtained would have been the same

as by that of the advancing front. On the other hand the time to reach 98 per cent saturation by THEWS' method is only just over a half that by the advancing front method, so that the value of D_{O_2} (THEWS) if calculated from $t_{98\%}$ would have come out nearly twice as high as

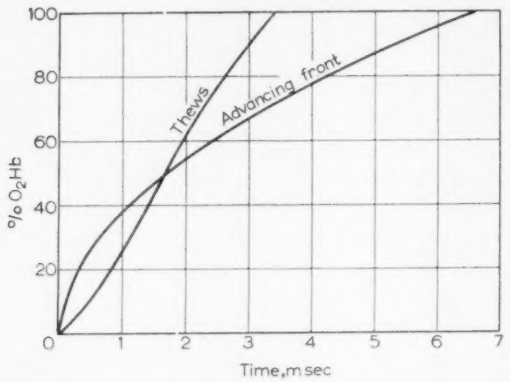

Fig. 12. Comparison of the rates of uptake of oxygen by a layer of 30 per cent Hb (w/v) solution 7×10^{-5} cm ($0{\cdot}7\mu$) thick when exposed to oxygen at 700 mm Hg pressure at 25°C, as calculated by THEWS' method and the advancing front method.

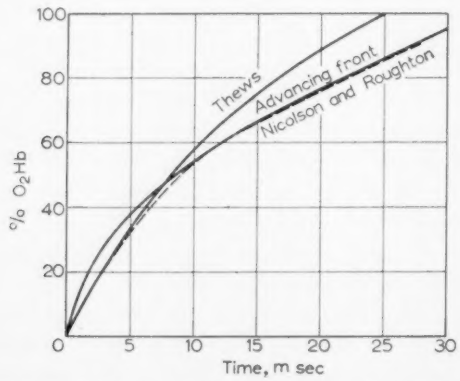

Fig. 13. Comparison of the rates of uptake of oxygen by a layer of 30 per cent Hb (w/v) solution 7×10^{-5} cm ($0{\cdot}7\mu$) thick when exposed to oxygen at 140 mm Hg pressure at 25°C, as calculated by the respective methods of THEWS, the advancing front and NICOLSON and ROUGHTON ($k' = 1{\cdot}7 \times 10^6$ M^{-1} sec^{-1}).

D_{O_2} (advancing front). It seems physically impossible that oxygen could under any circumstances diffuse at a faster rate into a medium containing a non-diffusing reactant than at that given by the advancing front formula: if this is so, the method of averaging used by THEWS has clearly led to a fallacious result.

Fig. 13 shows a similar comparison with all the quantities the same

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except the supernatant oxygen pressure, which in this case has been fixed at 140 mm Hg instead of at 700 mm Hg. There is again a coincidence at 50 per cent saturation but the discrepancy at 98 per cent saturation, though in the same direction as in Fig. 12, is now only about half as marked. Such variable behaviour shakes one's confidence further in the validity of THEWS' method. Fig. 13 also contains the result of a calculation under the same conditions by NICOLSON and ROUGHTON's (1951) method, the value of k' (the velocity constant of the reaction $O_2 + Hb \rightarrow O_2Hb$) being assumed to be $1{\cdot}7 \times 10^6$ M^{-1} sec^{-1}.

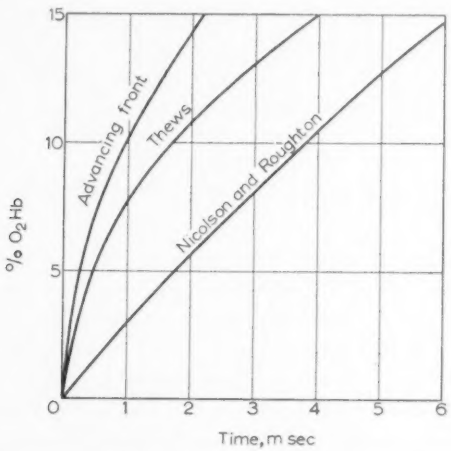

Fig. 14. Similar comparisons to those in Fig. 13 except that the oxygen pressure was 48 mm Hg and the temperature 38°C.

It is interesting to note how closely the Nicolson–Roughton curve and the advancing front curve line up with one another during the second half of the uptake process: this does indeed suggest that the advancing front method may be applicable to calculations on the rate of uptake of oxygen by partially saturated cells under the conditions prevailing in the lung capillaries (see the last section of this Review) provided a correction be inserted for the effect of the red cell membrane as can readily be done.

Finally Fig. 14 shows the initial rate of oxygen uptake by a 7×10^{-5} cm thick layer of 30 per cent (w/v) Hb solution when exposed to an oxygen pressure of 48 mm Hg at 37°C, as calculated by the advancing front method, the THEWS' method and the NICOLSON–ROUGHTON method. Only the last of these three methods is theoretically valid under these circumstances but the THEWS' method is less open to objection than previously, since at 48 mm Hg oxygen pressure and 37°C the haemoglobin at the gas–liquid interface will only reach an

equilibrium percentage saturation of about 80 per cent and furthermore the value of R will be much more nearly constant throughout the whole diffusion process than in the two preceding examples. Nevertheless the "Thews" rate in the early stages is much faster than the "Nicolson–Roughton" rate, and since the latter is calculated on physico-chemical principles which seem theoretically sound, the validity of the THEWS' method is again called in question. The reason that the THEWS' method gives too fast an initial rate in the present instance is that it is not justifiable under these circumstances to regard the speed of combination of oxygen with haemoglobin as infinitely fast in comparison with the diffusion process. The writer's final conclusion is that THEWS' method, able and ingenious though it is, must *always* be checked by some other method since it will be likely sometimes to give good results and sometimes to give bad results and there seems no *a priori* way of knowing in advance which way things will turn out.

2. *Diffusion plus chemical reaction velocity in membraneless spheres of haemoglobin solutions*

This subject is of particular interest in view of the well known tendency of mammalian red cells to "sphere," i.e. to change from their usual biconcave disc forms to that of spheres. It has often been argued that the biconcave form is superior as regards the rapid supply of oxygen to the haemoglobin of the red cell by diffusion (see HARTRIDGE, 1920; PONDER, 1925–1926) but calculations of the type given below throw some doubt on this tenet at any rate as regards sheep red cells.

For a sphere (radius s) of concentrated haemoglobin solution, in which D_{Hb} is negligible, the appropriate diffusion equation (see ROUGHTON, 1952) for penetration of oxygen from a gas phase, of partial oxygen pressure $= p_s$ is as follows:

$$D\frac{\partial^2}{\partial r^2}(rc) = \frac{\partial(rc)}{\partial t} + k'rc\,[\mathrm{Hb}] \qquad \ldots.(35)$$

subject to the boundary conditions

$$c = 0 \text{ at } t = 0 \text{ for } 0 \leq r < s$$

$c = c_s = \alpha p_s$ at $r = s$, at the surface of the sphere at all times;

c is finite at $r = 0$, at the centre of the sphere at all times.

The solution of this equation for the initial phase of O_2 uptake is

$$\overline{[\mathrm{O_2Hb}]} = 3c_s t\left[\frac{\sqrt{Dk'[\mathrm{Hb}]_0}\coth\left(s\sqrt{\frac{k'[\mathrm{Hb}]_0}{D}}\right)}{s} - s^2\right] \qquad \ldots.(36)$$

+ a transient series which rapidly becomes insignificant.

The full line in Fig. 15 gives the initial rate of O_2 uptake (as calculated from equation (36)) by spheres of 36 per cent (w/v) haemoglobin, of radii ranging from 1·5 to 3·0 μ at 37°C. This range covers the radii of normal sheep and human red cells, when sphered. The horizontal broken lines are the initial rates of O_2 uptake, at the same temperature and outside O_2 pressure, of layers of haemoglobin solution, of semi-thickness ranging from 0·5 to 1·0 μ. The greatest and least thickness of sheep and human red cells, in the biconcave disc form, fall within these limits. In these calculations k' was taken to be $1{\cdot}5 \times 10^6$

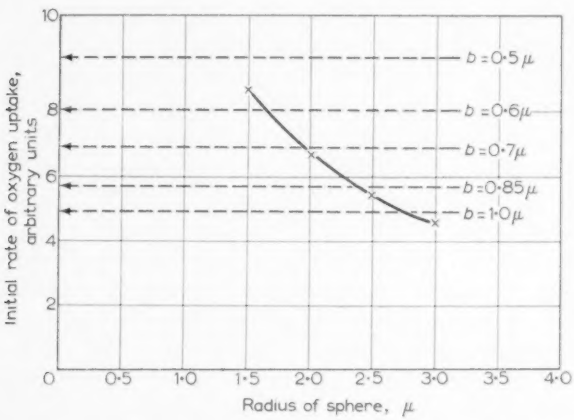

Fig. 15. Theoretical calculations of initial rate of oxygen uptake by membraneless layers of 36 per cent Hb solution (w/v) of varying thickness (horizontal broken lines) and membraneless spheres of the same Hb solution (full line) and of varying radii. Further details and discussion in text.

(M^{-1} sec^{-1}) and D to be $7{\cdot}2 \times 10^{-6}$ cm^2/sec. Similar calculations with CO gave practically the same relative results—the absolute rates were only about two-thirds as great, corresponding to the lower value of l' at 37°C, viz. $6{\cdot}7 \times 10^5$ (M^{-1} sec^{-1}).

ROUGHTON (1932) gave some reason for supposing that the biconcave sheep red cell could be taken as equivalent to a flat layer of semi-thickness equal to 0·7 μ, as regards calculations of gas uptake by simultaneous diffusion and chemical reaction. If his argument is correct, or roughly so, it would seem from Fig. 15 that there would be hardly any advantage, as regards speed of oxygen uptake, in the biconcave form of the sheep red cell, since the radius of the latter in the sphered form is about 2·0 μ and the initial rate of uptake by a sphere of this radius is practically the same as that by a layer of semi-thickness 0·7 μ. The human red cell is about twice the volume of the sheep red cell, and its radius when sphered is about 2·5 μ, instead of 2·0 μ. In

the case of human red cells Fig. 15 suggests that there might be about a 20 per cent advantage, from the diffusion plus chemical reaction velocity angle, in the cells being biconcave discs rather than spheres, but the advantage is decidedly less than has been generally supposed till recently. With red cells of still larger volume, the disadvantage of the spherical form would become more patent, whereas with smaller red cells than those of the sheep the spherical form would tend to be at a premium. Fig. 15 brings out the interesting point not hitherto made—so far as the writer knows—that the actual dimensions of sheep and human red cells may be in a critical region, wherein the exact shape—as regards speed of diffusion plus chemical reaction velocity—is relatively unimportant. Such a conclusion is, at first sight, strongly supported by the recent experimental determinations by CARLSEN and COMROE (1956, 1957) of the rates of uptake of CO by human red cells at 37°C, in the normal biconcave disc form and in the spherical form. No significant differences could be detected between the two forms; subsequently the same conclusion was reached with NO uptake.

In the Introduction, however, it has been already pointed out that diffusion through the red cell membrane (which is to be discussed in detail in the next main Sections of this article) is a limiting factor of the same order of magnitude, in the rate of oxygen uptake, as is diffusion plus chemical reaction in the interior of the red cell. It is still often stated (see e.g. HARRIS, 1955) that the surface area of the red cell in the biconcave disc form is as much as 9 times greater than its surface area when sphered. If this were true, then the experimental results of CARLSEN and COMROE would seem to entail a great increase in the permeability of the surface membrane to dissolved gases on sphering. According, however, to more recent calculations (*vide* HOFFMAN, 1958), this supposed nine-fold decrease in surface area of the red cell on sphering is a vast over-estimate, the actual decrease being only of the order of 40 per cent, not 900 per cent: on this basis, as shown later (pages 98 to 99), no significant change in permeability of the red cell membrane on sphering is required to explain the very interesting findings of CARLSEN and COMROE. In the writer's present view the biological *raison d'être* of the normal biconcave disc shape of the red cell may well have additional origins besides that of speeding up the rate of uptake of oxygen: possibly, for example, the disc form is better able to worm its way through partially closed blood capillaries in the intact circulatory system.

The calculated results have so far been limited to the initial phase of gas uptake, for which analytical solutions are available. Dr. A. KLUG has, however, carried out extended and very laborious computations on the relative rates of CO uptake at 37°C by discs of human haemoglobin (of semi-thickness = 0·7 μ) and spheres of the same volume,

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of radius 1·9 μ, over the range 0–40 per cent saturation. The two curves (see Fig. 16) were practically coincident over the whole range. This work has not yet been published in the ordinary literature and I am greatly indebted to Dr. KLUG for permitting me to quote it here.

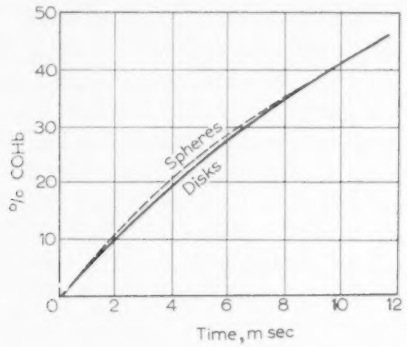

Fig. 16. Theoretical calculations by Dr. A. KLUG of the rate of uptake of carbon monoxide by disks (full line) and spheres (broken line) of haemoglobin solution, the spheres being of the same volume as the disks.

3. *Diffusion plus chemical reaction velocity in cylindrical systems*

The equations for cylinders suspended within an infinite medium of gas have been given by ROUGHTON (1952) and can also be obtained from the writings of other authors (see e.g. CRANK, 1956). The solutions of the equations contain Bessel Functions and will not be included here, since their only application to haemoglobin problems so far has been to the problem of the *raison d'être* of the red cell as a whole, not merely its shape. How would the animal body fare (see, e.g. BARCROFT, 1923) if the red cells of the blood were all lysed and the heterogeneous red cell-plasma system of normal circulating blood were thus replaced by a homogeneous haemoglobin solution of the same oxygen capacity as that of normal human blood? ROUGHTON's (1952) calculations suggest that, in addition to other physiological disadvantages, there might be an appreciable handicap as regards rate of oxygen uptake, since he finds that with open blood capillaries of radius 4 μ (the usual figure assumed in mammals) the rate of uptake of oxygen by a 15 per cent (w/v) solution of circulating haemoglobin should only be about one-third as great as that by normal red blood cells containing 30–35 per cent (w/v) haemoglobin.

Another cylindrical problem, of more practical physiological interest, is that of the diffusion of oxygen from the blood capillaries into surrounding muscular tissue, which in the case of red muscles like the diaphragm and the soleus contains the reversible oxygen carrier

myoglobin (muscle haemoglobin)—as well as the enzyme systems concerned in the ordinary metabolism of oxygen. The problem is essentially one of penetration from a central cylindrical core into a zone between two coaxial cylinders, wherein diffusion plus chemical reaction velocity occurs (KROGH, 1919). The equations for such processes, together with their initial analytical solutions, have been given in detail by ROUGHTON (1952) but in view of their complication and the fact that they are somewhat remote from the main theme of this article they will not be included here. Their most interesting application from the physiological point of view has been to MILLIKAN's (1937) observations on the reversible oxygen saturation and desaturation of myoglobin in the intact soleus muscle of anaesthetized cats. From these data ROUGHTON (1952) was able, with the aid of the appropriate diffusion plus chemical reaction equations, to calculate plausible values for the average distance between the patent blood capillaries in the intact soleus muscle (in the resting muscle the distance thus estimated was 100–150 μ).

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V. DIFFUSION PLUS CHEMICAL REACTION VELOCITY IN MEMBRANE-BOUNDED LAYERS OF HAEMOGLOBIN SOLUTION

The finding (depicted in Fig. 1), that the observed rate of oxygen uptake by sheep red cells is considerably less than the calculated rate

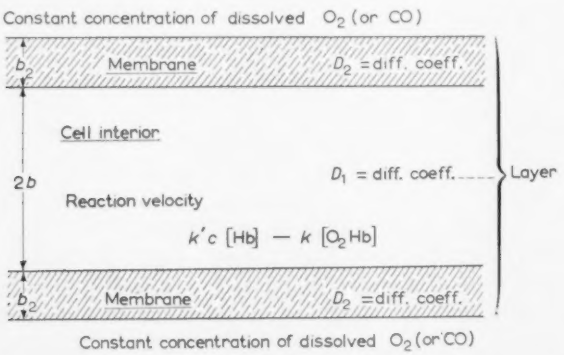

Fig. 17. Scheme of diffusion of dissolved gas into a membrane-bounded layer of haemoglobin solution.

for diffusion plus chemical reaction in the interior of the red cell, led NICOLSON and ROUGHTON (1951) to investigate quantitatively the effect of enclosing layers of concentrated haemoglobin in membranes of varying permeability. Fig. 17 illustrates schematically the nature of the problem. The equations to be solved are the same as in the case

of the membraneless layer for region 1 (i.e. equations (18) and (19)), and, in addition, in region 2, the equation

$$D_2 \frac{\partial^2 c''}{\partial x^2} = \frac{\partial c''}{\partial t} \qquad \ldots.(37)$$

where c'' is the concentration of dissolved gas in region 2. The boundary conditions are as follows:

$$c = c'' = 0 \text{ for } \left\{ \begin{array}{l} x < b_1 + b_2 \text{ if } x \text{ is positive} \\ -x < b_1 + b_2 \text{ if } x \text{ is negative} \end{array} \right\} \text{ at } t = 0$$

$$[\mathrm{Hb}] = [\mathrm{Hb}]_0 \text{ for } \left\{ \begin{array}{l} x < b_1 \text{ if } x \text{ is positive} \\ -x < b_1 \text{ if } x \text{ is negative} \end{array} \right\}$$

$$c = c_b \text{ at } x = b_1 + b_2 \text{ for all times}$$

$$\left. \begin{array}{c} c = c'' \\ D_1 \dfrac{\partial c}{\partial x} = D_2 \dfrac{\partial c''}{\partial x} \end{array} \right\} \text{ at } x = b_1 \text{ for all times}$$

For the initial phase, analytical solutions are again available (see also Danckwertz, 1950, 1951; Crank, 1956, 1957) the oxygen uptake in chemical combination up to a short time, t, being given by

$$[\overline{\mathrm{O_2Hb}}] = k' c_b [\mathrm{Hb}]_0 t \frac{\tanh \omega}{\omega} \frac{1}{1 + \dfrac{\omega}{\lambda} \tanh \omega} \qquad \ldots.(38.1)$$

where

$$\omega = b_1 \sqrt{\frac{k' [\mathrm{Hb}]_0}{D_1}} \qquad \ldots.(38.2)$$

$$\lambda = \frac{D_2}{b_2} \Big/ \frac{D_1}{b_1} \qquad \ldots.(38.3)$$

In the case of the membraneless layer, in which $\lambda = \infty$ equation (38.1) simplifies to

$$[\overline{\mathrm{O_2Hb}}] = k' c_b [\mathrm{Hb}]_0 t \frac{\tanh \omega}{\omega} \qquad \ldots.(39)$$

which is identical with equation (25) if the transient term in the latter is neglected.

If D_1 is very large, ω becomes very small and since the value of $(\tanh \omega)/\omega$ then tends to unity, equation (39) becomes—as would be expected—simply the equation for the initial combination of oxygen with haemoglobin in homogeneous solution, viz.

$$\overline{\mathrm{O_2Hb}} = k' c_b [\mathrm{Hb}]_0 t \qquad \ldots.(40)$$

The effect of diffusion plus chemical reaction velocity in the cell interior is thus equivalent to a reduction in the homogeneous chemical reaction rate by a factor $(\tanh \omega)/\omega$, whilst the addition of a membrane produces a further reduction by a factor $1/(1 + \frac{\omega}{\lambda} \tanh \omega)$. This way of looking at the matter will perhaps enable the reader to appreciate more clearly the effects of these two diffusion factors in the overall rate of uptake by the red cells of gases, which combine chemically with the haemoglobin therein.

It is also useful to consider the limiting case, in which k' tends to infinity and the penetrating gas is instantly combined once it has passed through the red cell membrane. From equation (38.2) it follows that ω also tends to infinity and since $\tanh \omega$ then becomes unity equation (39) simplifies to

$$[\overline{O_2Hb}] = k' c_b [Hb]_0 t\lambda/\omega^2$$

$$= \frac{c_b D_2}{b_2 b_1} t \qquad \ldots.(41)$$

The total uptake per unit area of the layer then

$$= b_1 [\overline{O_2Hb}]$$

$$= \frac{c_b D_2}{b_2} t \qquad \ldots.(42)$$

which is simply the equation for the passage of gas through unit area of a membrane of diffusion coefficient D_2 and thickness b_2, or of permeability P_2 where $P_2 = D_2/b_2$.

At fixed values of k', c_b, $[Hb]_0$, b_1, D_1 and hence of ω, equation (38.1) may be transformed to the following

$$\frac{t}{[\overline{O_2Hb}]/[Hb]_0} = A + \frac{B}{\lambda} \qquad \ldots.(43.1)$$

where

$$A = \frac{\omega}{k' c_b \tanh \omega} \qquad \ldots.(43.2)$$

$$B = \frac{\omega^2}{k' c_b} \qquad \ldots.(43.3)$$

The time divided by the fractional saturation reached during the initial period, when plotted against $1/\lambda$, should thus give a straight line of slope equal to $\omega^2/k'c_b$. From such a graph the value of $1/\lambda$ can at once be read off, if the initial rate of saturation of the red cell suspension has been measured experimentally and k' has been determined by a separate experiment on the rate of combination of oxygen with

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haemoglobin in homogeneous solution, the value of ω being calculable from k' assuming the values of D_1 and b_1 which have already been used. FORSTER *et al.* (1957) have recently determined λ in this way for human red cell suspensions in Ringer–Locke solution at 37°C, using for this purpose the two processes $O_2 + Hb \rightarrow O_2Hb$ and $CO + Hb \rightarrow COHb$. ROUGHTON *et al.* (1957) have likewise estimated λ under similar conditions from data on the displacement reaction $CO + O_2Hb \rightarrow O_2 + COHb$. The experimental results will be discussed in the next subsection, together with a reference to similar work on sheep red cell suspensions at 15–20°C. The application of the human data to physiological problems of gas exchange in the human lung will be described in the next main section of this article.

1. *Estimation of the ratio of the permeabilities of the red cell membrane and of the red cell interior*

The apparatus used during the last four years for this purpose is fully described by FORSTER *et al.* (1957) and is depicted in Fig. 18. It is

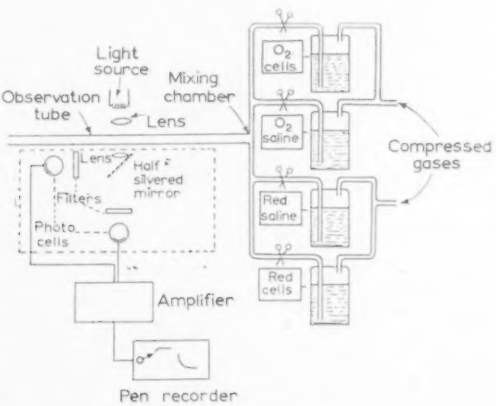

Fig. 18. Diagram of photo-electronic apparatus of FORSTER *et al.* (1957) for measuring rate of uptake of dissolved gases by red cell suspensions. For full description, see original paper.

essentially the same in principle as that of HARTRIDGE and ROUGHTON (1927), the main improvement being in the measurement of the percentage saturation of the red cells in the fluid streaming down the observation tube. By modern photo-electronic methods it has proved possible to increase the accuracy two to three times over that attainable by the visual Hartridge Reversion Spectroscope even in its optimal range, i.e. 30–70 per cent saturation. The Reversion Spectroscope was, furthermore, inapplicable at the beginning of the process (0–20 per cent saturation), which is by far the most satisfactory range for study

from the theoretical point of view, since the "initial" analytical solutions are reasonably valid here. The photo-electronic method, on the other hand, is just as available in the initial range as at later stages. The main difficulty with the photo-electronic method lay in the "noise" due to irregular light scattering by the refractile walls of the red cells: this difficulty was, however, completely overcome by a modification of the differential method, which MILLIKAN (1937) was the first to apply to the study of kinetics of haemoglobin in solution. It consists simply in focusing the examining beam of light, after it has passed through the observation tube, on to a half-silvered mirror, thus

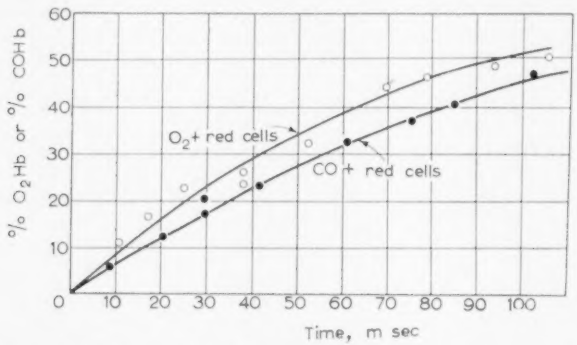

Fig. 19. Rate of uptake of O_2 and of CO by a suspension of human red cells at 37°C. The initial concentration of dissolved gas was 9×10^{-5}M, and the mixed red cell suspension contained 1 part of whole blood in 79 parts of saline.

splitting the light into two beams, one of which then passes through an interference filter with peak transmission at e.g. 549 mμ before falling on a photo-cell, whereas the other beam passes through a second interference filter with peak transmission at a neighbouring wavelength, e.g. 564 mμ, before falling on a second matched photo-cell. The difference between the two photocurrents, after conventional amplification, is registered with a pen recorder, from the readings of which the percentage saturation of the red cells can readily be calculated. The particular peak transmissions were chosen so that in one case there was a significant difference in light absorption, as the haemoglobin became saturated with gas, whereas in the other case there was practically no change, the peak wavelength being isobestic for reduced haemoglobin and for oxyhaemoglobin or carboxyhaemoglobin, as the case might be. In both cases, however, there was scattering of light by refraction but the amount thereof was much about the same and the effect of the scattered light was therefore practically eliminated by making the two photocells "fight" against each other.

Typical results by this method, which is usually accurate to within about $\pm$ 2 per cent saturation, are shown in Fig. 19 for uptake of O_2

and uptake of CO by human red cells at 37°C. It is clear that the initial rates of uptake can be deduced with reasonable precision from such curves.

Table 4 shows how closely the average values of λ, as calculated from measurements on four different processes, agree with one another in

TABLE 4

Average Values of λ for Human Red Cells Suspended in Ringer–Locke Solution at 37°C

Process	*Average value of λ*
$O_2 + Hb \rightarrow O_2Hb$	$1{\cdot}5_3$
$CO + Hb \rightarrow COHb$	$1{\cdot}5_6$
$NO + Hb \rightarrow NOHb$	1·5
$CO + O_2Hb \rightarrow O_2 + COHb$	$1{\cdot}5_3$

Heparinised blood samples from 10 normal adults were used for the experiments. The final dilution of blood in the reacting fluid was usually 1 in 80. For the process $O_2 + Hb \rightarrow O_2Hb$ FORSTER *et al.* (1957) gave a figure of $\lambda = 1{\cdot}44$, which was based on a value for k' of $3{\cdot}5 \times 10^6$ M^{-1} sec^{-1}. Recent work by GIBSON and ROUGHTON (1958) has, however, shown that the extrapolated value of k' at zero time is only about half as large. The value of $\lambda = 1{\cdot}53$ in the Table is based upon average values for k' and k_c', at zero time, of $1{\cdot}8 \times 10^6$ M^{-1} sec^{-1} and $9{\cdot}2 \times 10^4$ M^{-1} sec^{-1} respectively (as given in Table 1 of the present review).

the case of human red cell suspensions at 37°C. It should, however, be mentioned that the values of λ in individual experiments showed a far larger scatter in the case of the process $CO + O_2Hb \rightarrow O_2 + COHb$ than in the case of the other processes. The reason for the larger scatter is that in the case of the process $CO + O_2Hb \rightarrow O_2 + COHb$ there is a much smaller difference between the rates in red cell suspensions and in homogeneous haemoglobin solutions, so that a slight error in the determination of either will have proportionally a far bigger effect in the calculation of λ.

Higher values of λ were observed by ROUGHTON and SIRS (1955) in the case of sheep red cell suspensions at 18°C (about) and in addition an apparent seasonal effect appeared. The bloods from four experimental rams (thanks for which is due to the Cambridge University Animal Research Station) were worked on in February, whilst the animals were living indoors, and later again in May of the same year after the animals had been put out to grass. The "winter" blood gave an average value for λ of 2·5 (by NO uptake and O_2 uptake experiments) whereas for the "spring" blood of the same animals λ was found to be about 7·5, i.e. a three-fold—and certainly significant—increase. Variations of pH within the range pH 7–8 seemed to be without any marked effect.

There is obviously need for a study of λ over a wide range of conditions, physiological and pathological. It would, for example, be very interesting to examine the effect on λ of the polycythaemia in blood, which occurs in the course of adaptation to high altitudes. It would be natural to expect an increase in λ under such conditions, but tests have yet to be made. For such purposes it would be a great advantage if it were possible to extend to red cell suspensions some

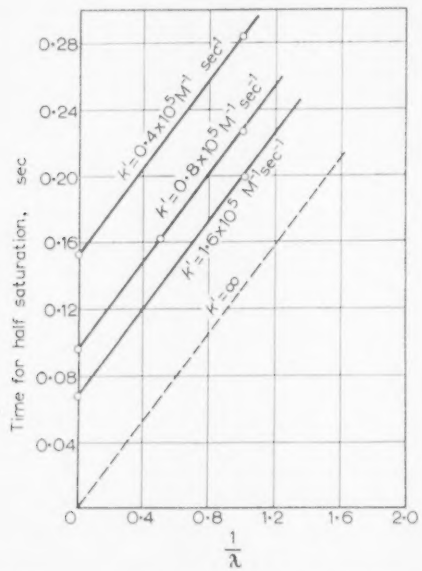

Fig. 20. Relation between $1/\lambda$ and τ, the time to reach half saturation inside the red cell, for three reactions of varying rate. The broken line is based on the equations given on page 257 of the paper by NICOLSON and ROUGHTON (1951) and corresponds to $k' = \infty$.

adaptation of GIBSON's stopped flow method, which has proved so successful (see Chapter 1, pp. 9–11) in the measurement of the kinetics of haemoglobin in homogeneous solution. Attempts on these lines are in progress in the writer's laboratory at the moment of writing (December, 1957).

So far λ has been determined from the initial rate of uptake of reacting gases by red cell suspensions. NICOLSON and ROUGHTON (1952) also carried out numerical computations on membrane-bounded layers of concentrated haemoglobin solution over a much more extended range. They found that the time for half-saturation is linearly related to $1/\lambda$ just as is the time for an initial small increase in saturation, according to equations (38.1) and (43.1). Fig. 20 gives a plot of τ (the

time for half-saturation) against $1/\lambda$ for three reactions of varying rate, but of the same order as the rate of the process CO + Hb → COHb. The effect of the membrane is quite clearly additive to the effect of diffusion plus chemical reaction velocity within the red cell. Fig. 21 shows a generalization of these results, the relation between $1/\lambda$ and $\tau \times (D_1/b_1^2)$ being plotted for various values of the parameter $k'[\mathrm{Hb}]_0 b^2/D_1$, these values being printed against the respective curves.

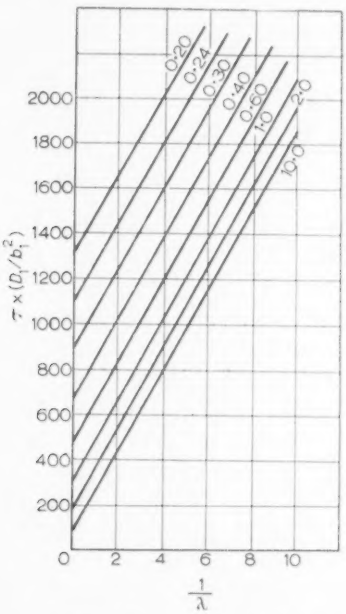

Fig. 21. Relation between $1/\lambda$ and $\tau \times (D_1/b_1^2)$, the generalized parameter referred to in the text. Figures proportional to this parameter are printed against the respective curves. For fuller explanation see NICOLSON and ROUGHTON (1951).

The actual value of D_2, the diffusion coefficient of the dissolved gas in the red cell membrane, could be calculated from λ, if the values of D_1, b_1 and b_2 were known. The values of D_1 and b_1, viz. 7×10^{-6} cm^2/sec and 7×10^{-5} cms respectively, used so far, are not likely to be far out, but estimates of b_2 vary all the way from 5×10^{-7} to 5×10^{-6} cms. For $b_2 = 5 \times 10^{-6}$ cms and $\lambda = 1{\cdot}5$, D_2/D_1 works out at 0·11 whereas for $b_2 = 5 \times 10^{-7}$, D_2/D_1 equals 0·011.

2. *Diffusion plus chemical reaction velocity in membrane-bounded spheres*

The effect of a membrane can be treated in a similar way to that which has just been adopted in the case of layer diffusion. The initial rate of

gas uptake by a membraneless sphere of haemoglobin solution, of radius s and diffusion coefficient D_1, has already been given in equation (36): the effect of a bounding membrane of diffusion coefficient D_{2s} and thickness b_{2s} is to reduce this by a factor, ψ_s given by

$$\psi_s = \frac{1}{1 - \frac{1}{\lambda_s} + \frac{\omega_s}{\lambda_s} \coth(\omega_s)} \qquad \ldots.(44.1)$$

where

$$\omega_s = s\sqrt{\frac{k'[\mathrm{Hb}]_0}{D_1}} \qquad \ldots.(44.2)$$

$$\lambda_s = \frac{D_{2s}}{b_{2s}} \Big/ \frac{D_1}{s} \qquad \ldots.(44.3)$$

Equation (44.1) can readily be deduced from equations (8.58) and (8.60) of CRANK's (1956) *The Mathematics of Diffusion*, Chapter VIII. To illustrate its use we shall now discuss more quantitatively the effect of the cell membrane in CARLSEN and COMROE's (1956, 1957) experiments on sphered human red cells to which reference has already been made. Sphering of the red cell in isotonic solution causes no change in its total volume and we shall assume tentatively, as a first approximation, that:

(*a*) The volume of the surface membrane of the red cell is also unchanged by sphering. If the surface area of the sphered red cell is about 0·7 times the surface area of the red cell in its normal biconcave disc form it then follows that b_{2s} equals $1{\cdot}4b_2$.

(*b*) The value of D_1 in the interior of the sphered red cell membrane is the same as that in the interior of the normal red cell.

(*c*) $s = 2{\cdot}5\,\mu = 2{\cdot}5 \times 10^{-4}$ cm, $b_1 = 7 \times 10^{-5}$ cm.

(*d*) $k' = 1{\cdot}5 \times 10^6\ \mathrm{M^{-1}\,sec^{-1}}$, $[\mathrm{Hb}]_0 = 2 \times 10^{-2}$ M, so that $\omega_s = 16{\cdot}3$.

On these assumptions

$$\lambda_s = \frac{D_{2s}}{b_{2s}} \Big/ \frac{D_1}{s} = \frac{D_{2s}}{D_2}\frac{b_2}{b_{2s}}\frac{s}{b_1}\left(\frac{D_2}{b_2} \Big/ \frac{D_1}{b_1}\right) = 3{\cdot}57\,\frac{D_{2s}}{D_2}\,\lambda$$

Thus

$$\psi_s = \frac{1}{1 + \frac{4{\cdot}3}{\lambda}\,\frac{D_2}{D_{2s}}}$$

If $\lambda = 1{\cdot}5$ as previously

$$\psi_s = \frac{1}{1 + 2{\cdot}85\,\frac{D_2}{D_{2s}}}$$

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For the layer, the analogous reduction factor, ψ, due to addition of a membrane is given by the equation

$$\psi = \frac{1}{1 + \frac{\omega}{\lambda} \tanh \omega}$$

where $\omega = b_1 \sqrt{\frac{k' [\mathrm{Hb}]_0}{D_1}} = 4{\cdot}6$ for the present values of k', $[\mathrm{Hb}]_0$ and D_1, so that with $\lambda = 1{\cdot}5$

$$\psi = 0{\cdot}25$$

CARLSEN and COMROE's work suggests that ψ_s is approximately equal to ψ, which will be the case in the above example if $D_{2s} = 0{\cdot}95 D_2$, i.e. an insignificant difference.

On these simplifying assumptions, CARLSEN and COMROE's finding that sphering of the human red cell leads to no significant change in the overall rate of gas uptake *in vitro* is to be expected if there is no appreciable change in the permeability of the red cell membrane on sphering.

VI. Diffusion and Chemical Reaction Velocity in the Circulating Blood as Limiting Factors in the Rate of Uptake of Oxygen and Carbon Monoxide in the Lung

The transport of O_2 or CO molecules from the smallest air cells of the lungs—the alveoli—to the haemoglobin molecules of the red cells in the blood capillaries of the lung can be broken down into four successive processes:

(*a*) Diffusion through the alveolar epithelium and the walls of the blood capillaries.

(*b*) Passage through the blood plasma which separates the walls of the blood capillaries from the surfaces of the red cells. This process is a mixture of molecular diffusion plus an—at present—unassessable amount of mixing in the plasma due to the hydrodynamic conditions in the capillaries.

(*c*) Diffusion through the red cell membrane.

(*d*) Diffusion plus chemical reaction velocity in the interior of the red cell.

Processes (*c*) and (*d*) have already been discussed fairly exhaustively in the present review and it now remains to consider how important they are as limiting factors in the whole chain of processes involved in uptake of O_2 and/or CO in the lung.

Previous to the work of HARTRIDGE and ROUGHTON (1923), it had generally been assumed by physiologists—either tacitly or explicitly—that processes (*c*) and (*d*) were too fast to have any appreciable limiting

effect on the overall rate of uptake of O_2 and/or CO in the lung. Once either of these gases had entered the lung plasma it was supposed that they "instantaneously" reached equilibrium with the haemoglobin of the red cells. The partial pressure of dissolved O_2 (or CO) in the blood plasma at any point along the blood capillary could therefore be calculated from the dissociation curve of haemoglobin and the haemoglobin saturation at that point. The diffusion constant of the lung, D_L, was defined by BOHR (1909) and KROGH (1909, 1914) in accordance with the equation

$$(P_a - P_m)\, D_L = V_g/t \qquad \ldots.(45)$$

where P_a is the partial pressure of O_2 (or CO) in the lung alveoli in mm Hg, P_m is the average partial pressure of the gas in the lung capillary (on the assumption of complete equilibrium with the haemoglobin at every point) and V_g is the volume of gas in ml taken up in t minutes. In recent years the expression "diffusion constant of the lung" has been replaced by "diffusing capacity of the lung," but whichever term was used it was assumed that D_L was a function only of the properties of the pulmonary membrane separating the alveolar air from the plasma in the lung capillaries, being in fact equal to $d_{O_2} A/x$ where d_{O_2} (or d_{CO}) is the diffusion coefficient of the pulmonary membrane to the dissolved gas, A is the total area and x is the average thickness of the pulmonary membrane. Actually since the time of BOHR (1909), D_L has always been measured directly either for O_2 or more commonly for CO, its indirect calculation being too uncertain owing to the difficulty of obtaining reliable values of A and x.

One of the prime physiological incentives which led HARTRIDGE and ROUGHTON to embark on their rapid reaction velocity studies was the urge to find out whether processes (*c*) and (*d*) did in fact have any limiting effects on the rate of gas uptake in the lung and, if so, how much. Their own preliminary researches (*vide* ROUGHTON, 1925) pointed definitely to a limiting effect of these processes: this indication has recently been confirmed and placed on a fairly sound quantitative basis by the work of FORSTER and ROUGHTON and their collaborators (FORSTER *et al.*, 1957; ROUGHTON *et al.*, 1957; FORSTER *et al.*, 1957; ROUGHTON and FORSTER, 1957; see also ROUGHTON, 1945; KRUHØFFER, 1954; GIBSON *et al.*, 1955). The problem has also been very lately reviewed in a most lucid fashion by FORSTER (1957). As the result of these studies the following equation has now emerged

$$\frac{1}{D_L} = \frac{1}{D_M} + \frac{1}{\theta V_c} \qquad \ldots.(46)$$

where D_L = the overall diffusing capacity of the lung as calculated, with the aid of equation (45), from measurements of

the rate of gas uptake, and includes both a pulmonary membrane component and a red cell component,

D_M = the diffusing capacity of the pulmonary membrane $\left(= d_{O_2}\frac{A}{x}$ or $d_{CO}\frac{A}{x}\right)$ plus some component due to the blood plasma if there are any appreciable gradients in the latter due to incomplete mixing of the plasma in the circulating blood.

V_c = the total volume of blood in the lung capillaries.

θ = the rate of uptake of gas by the red cells expressed as ml gas taken up, per ml of whole blood of known haemoglobin concentration, per minute per mm Hg difference of partial pressure between the plasma and the interior of the red cells. It is assumed that θ can be obtained from the *in vitro* measurements on red cell suspensions already described in this review.

The theoretical derivation of equation (46) has been given by ROUGHTON and FORSTER (1957): experimentally it has been confirmed

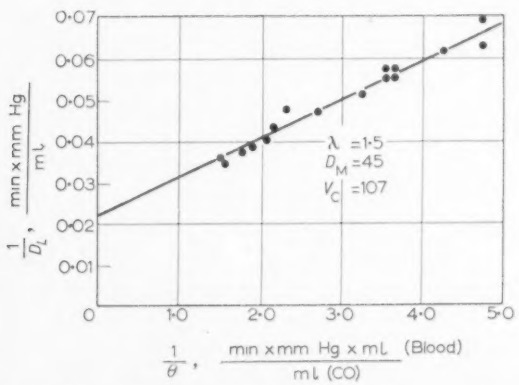

Fig. 22. Relation between the reciprocal of D_L for CO, as measured experimentally at various oxygen pressures and the reciprocal of θ, assuming $1/\lambda = 1{\cdot}5$.

by measurements of D_L for CO—on normal human subjects—at varying alveolar oxygen pressures, obtained by inspiration of gas mixtures containing small percentages of CO plus O_2 at partial pressures of the latter ranging usually from 150 to 750 mm Hg (KRUHØFFER, 1954; FORSTER *et al.*, 1957). The value of θ which is, of course, proportional to $\mathscr{M}'_c$ (*vide* Table 1), was estimated experimentally over this range of oxygen pressures from *in vitro* determinations of the rate of displacement of O_2 by CO in red cell suspensions, prepared from the blood of some of the human subjects used for the measurements of D_L. Fig. 22

shows a plot of $1/D_L$ versus $1/\theta$ for a typical case of a normal man at rest: the points fall satisfactorily on a straight line (as equation (46) requires); the intercept of the line on the vertical axis gives the value of $1/D_M$, whilst its slope gives the value of V_c the total volume of blood in the blood capillaries of the living human lung—an interesting quantity which had not been estimated by physiologists prior to the work of ROUGHTON (1945). In the present case $D_M = 45$ ml/min/mm Hg, i.e. about 1·9 times the value of D_L for CO when breathing air at atmospheric pressure, whilst $V_c = 107$ ml. On the average (of a large number of determinations of D_L at rest by varying methods), D_L (breathing air) = 25 ml/min/mm Hg, $D_M = 53$ ml/min/mm Hg and $V_c = 78$ ml. The fact that D_L (for CO, breathing air) is about one-half D_M means that the intracapillary "resistance" to the passage of CO is about one-half of the total resistance. When breathing oxygen at 1 atm pressure, on the other hand, D_L for CO is only about one-fifth of the value of D_M, so that in this case the intracapillary "resistance" is about 80 per cent of the total resistance, due to the much slower rate of replacement of O_2 by CO in the red cell at high oxygen pressures. Although there are still some uncertain features—both experimental and theoretical—in the present treatment there can be little doubt that gradients within the blood itself do exert significant limiting effects on the uptake of carbon monoxide in the human lung.

In an average normal man the pulmonary blood flow under resting conditions is about 100 ml/sec: this means that the average time spent by the blood in passing through the lung capillaries, t_c, is $V_c \div 100$, i.e. 0·78 sec. This figure is several times higher than that observed directly by VOGEL (1947) in experiments on the lungs of anaesthetized cats. Part of the discrepancy might be due to the difference between the sizes of men and cats, but it would be interesting, if possible to apply the two methods to the same animal.

Carbon monoxide (the use of which in this field was first suggested by BOHR (1909)) has proved a uniquely valuable gas for "calibrating" the human lung under a wide range of conditions—normal and pathological; it is assumed that the value of D_L for oxygen can be obtained from the value of D_L for CO, by multiplying the latter by a factor of 1·23, this quantity being deduced from the measurements of KROGH (1919) on the ratio of the diffusion coefficients of isolated mammalian tissues to oxygen and carbon monoxide respectively. The value of D_L for O_2 has only been obtained directly up to the present at low alveolar oxygen pressures (i.e. of the order of 60 mm Hg and less) and this is one of the reasons why equation (46), which is theoretically just as valid for O_2 as for CO, has not yet been tested for oxygen. The analogous values of θ for uptake of O_2 by partially saturated red cells under the conditions actually prevailing along the lung capillaries are, furthermore,

more difficult to obtain and less certainly known than are the values of θ for the replacement of O_2 by CO: it is hoped, however, that current work in Dr. FORSTER's laboratory at Philadelphia with the recently developed oxygen electrode of CLARK (1956) may provide the requisite values of θ for O_2 uptake. Possibly also the problem may be accessible to the "advancing front" methods discussed earlier (see pp. 66–72 and 84). There is, however, little reason to doubt that, in the physiologically more interesting and important case of oxygen uptake in the lung, the limiting effect of intracapillary resistance is at least of the same order of magnitude as in the case of carbon monoxide uptake. The average normal resting values for D_L, as measured directly at low oxygen pressures, are 21 ml/min/mm Hg whereas D_M for O_2 if taken to be equal to 1·23 × the average value of D_M for CO is 1·23 × 53 = 65 ml/min/mm Hg. On this basis the intracapillary resistance to oxygen works out at about two-thirds of the total resistance, and at about twice the resistance of the pulmonary membrane resistance (the latter including, as above mentioned, any constituent due to gradients in the blood plasma).

The theoretical and experimental methods described in this section have so far been tested in the main on normal human beings at rest, but they are now being actively applied in many physiological and clinical laboratories all over the world to other conditions, both normal and pathological. If the writer may end on a somewhat personal note, he would say that it has been a particular satisfaction to him to witness —and to take some part in—the development of this subject from its early, rather severely mathematical and physico-chemical origins, to its present field of interest which is very largely centred in laboratories of clinical physiology.

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THE HYDRODYNAMICS OF THE ARTERIAL CIRCULATION

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THE HYDRODYNAMICS OF THE ARTERIAL CIRCULATION

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I. Introductory Survey

The last decade or so has seen the widespread application of accurate methods for recording arterial pressure pulsations, and there is promise that in the near future reliable techniques will become more generally available for the measurement of pulsatile arterial blood-flow. The question therefore arises as to the interpretation of the results which these methods give us. As far as non-pulsatile blood-flow is concerned, the theoretical background is considerable, and although some problems of anomalous viscosity remain to be explored, the lines of approach already available appear to be sufficiently established. On the other hand, the situation with respect to phasic or oscillatory flow is by no means so distinct. If the interpretation of experimental data is not to be purely qualitative and empirical, it is essential that some coherent physical theory of the arterial circulatory events should be available. However, neither the standard textbooks of physiology nor those of hydrodynamics contain the necessary theoretical background to the problem, and it must be sought in a number of publications scattered in the literature. The "Windkessel" model and theory of the arterial system has been the basis of most textbook descriptions for fifty years, but we venture to suggest that the alternative approach presented here may prove more adaptable to present needs. The emphasis of the Windkessel theory is on the intermittent and transient nature of the pressure and flow pulses in the arterial system, and on close examination does not appear sufficiently unrestricted in its basic assumptions to be capable of general application.

The "steady-state" approach which we advocate emphasises the repetitive and cyclic nature of arterial flow and pressure, and thus has many fruitful analogies in alternating-current theory. This resolution of pulsatile events into oscillatory series has from time to time in the past been employed in haemodynamic studies, and it is surprising that the method has not yet achieved the wide currency which we feel is its due; our principal aim here is to direct renewed attention to it. The experimental evidence so far available may seem at times to be somewhat incomplete, but the approach appears to be extremely promising

and affords a compact method of utilizing the experimental data which modern instruments can now provide.

The historical background

William Harvey published his classical work *De Motu Cordis et Sanguinis* in 1628 (translated Franklin, 1957) and demonstrated the fact that the heart pumped blood into the arteries, so that it was distributed throughout the body and returned to the heart by the veins. This initiated modern cardiovascular physiology, and it was relatively soon after this that Stephen Hales, around 1700, started his investigations into the physical aspects of the circulation. It seems clear from our knowledge of his writings that he was solely interested in this topic as an application of the new advances in physics which had taken place in the previous years; he was working in Cambridge when Newton was still a Fellow of Trinity. He is largely remembered as the man who first measured the arterial and venous blood pressures, but he also made valuable observations on the measurement of cardiac output, introduced the concept of the peripheral resistance in arterioles and discussed the functional significance of the elastic arterial walls.

The next work of significance in the physical analysis of the circulation began about 1830 with the researches of Poiseuille on the laws governing the flow of liquids through cylindrical pipes, and a few years later, of the Weber brothers (especially E. H. Weber) on the transmission of the pulse-wave. Here we enter the modern era. The importance of Poiseuille's work in the progress of hydrodynamics needs no emphasis, but it is pertinent to remark here that he was a physician who engaged in his physical researches in an attempt to elucidate the physiological problems of the circulation. Apart from the formula describing steady laminar flow in cylindrical tubes, on which his fame rests, the earliest of his published works (1828) dealt with the volume-changes in arteries due to the pulse. His technique foreshadowed the modern methods of plethysmography (Barcroft and Swan, 1953). His microscopical observations on the distribution of the red corpuscles in small vessels led him to the concept of what we call the velocity-profile (Herrick, 1942), and he also originated the use of the U-tube mercury manometer in physiology. It is somewhat ironic that although the knowledge of Poiseuille's formula is of qualitative interest in understanding the physical problems of the circulation, there is virtually no part of the system to which in life it can be applied precisely. In the smallest vessels, in which Poiseuille was so interested, considerable deviation occurs because blood, being a suspension of particles (the red corpuscles) behaves approximately as a Newtonian fluid only in tubes of radius greater than 200 μ. In larger vessels where we can assume orthodox viscous behaviour, the flow is oscillatory

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and not steady (all arteries and the larger veins). The form of POISEUILLE's equation with which we are familiar

$$Q = \frac{\pi R^4 (P_1 - P_2)}{8 \mu L} \qquad \ldots . (1)$$

(Q = flux, or rate of volume flow; P_1 and P_2 are the hydrostatic pressures at either end of the tube whose length is L and radius is R, while μ is the coefficient of viscosity of the liquid) is due to the mathematical derivation by HAGENBACH some 18 years after POISEUILLE published his empirical relation in 1842. (See HATSCHEK (1928) for a full review.)

The work of E. H. WEBER attempted to relate the velocity of the pulse-wave in the arterial tree, which is initiated by each cardiac impulse, with its elastic properties. The first work on this subject (LAMBOSSY, 1950, 1951) appears to be that of YOUNG (1808, 1809). This pioneer work was carried much further by MOENS, in a monograph in 1878. Contemporaneously, although apparently independently, this problem was solved by KORTEWEG, who developed some earlier work of RÉSAL (LAMB, 1932). The solution is commonly known as the MOENS–KORTEWEG formula, for the velocity of wave-transmission in an incompressible, non-viscous fluid, enclosed in a thin-walled elastic tube

$$c_0 = \sqrt{(Eh/2R\rho)} \qquad \ldots . (2)$$

(c_0 = wave-velocity; E = Young's Modulus of the wall of thickness h; R = mean radius of the tube; ρ is the density of the liquid.)

Further discussion of this topic is deferred to Section III; one may remark, however, on the strange fact that although the idea of propagation of disturbances in the arterial system has been long established, a vast body of work has been concentrated almost entirely in attempting to treat its behaviour as a whole, neglecting propagation. It is obvious that when dealing with a system of such peculiar and complex properties, many simplifying assumptions must be made to start a theoretical analysis; but past experience has shown that simplifications made explicitly as an analogy tend to be inherited by successive workers, until the abstractions of the analogue have been taken for reality. This criticism is in particular levelled at a great deal of the work on what is usually known as the Windkessel theory. This particular model has so dominated work on the physical behaviour of the circulation since 1900 that it must be considered separately, although no complete review of its extensive literature can be included here.

1. *The Windkessel model of the arterial tree*

The demonstration that arteries expand in time with the heart-beat dates from antiquity, although HARVEY was the first to demonstrate

that the expansion was due to the ejection of blood by the contraction of the ventricles of the heart. He drew attention to the fact, although it must long have been familiar to surgeons, that a wound in an artery caused an intermittent squirt of blood, while on the other hand the flow of blood from the smallest vessels, such as in a cut in the skin, was continuous and not pulsatile. Stephen Hales (1733) was the first to point out that this conversion of a pulsatile ejection by the heart into a steady flow in the capillaries was due to the elastic properties of the arterial walls. At the end of cardiac systole, the arteries hold an increased volume of blood, with a consequent increase in pressure, while during diastole, the phase of the cardiac cycle when the heart is refilling, the elastic tension in the walls of the arterial tree continues to force blood through the capillary bed. Regurgitation into the heart is, of course, prevented by the valves at the inlet to the aorta. Hales referred to this function of the arteries as that of an elastic reservoir.

In the German literature this concept of an elastic reservoir has been termed the Windkessel, likening the storage function of the arterial tree to that of an air-chamber of a force-pump, such as were found on fire-engines. As a descriptive analogy this is undoubtedly of value, but like other analogies it can be pressed too far, as we consider it has been. The reader will find a very full discussion in the monograph of Wezler and Boger (1939). The monograph by Apéria (1940) also contains an interesting historical section, although the work itself has been criticized by Broemser *et al.* (1943). The papers of Wetterer (1956) and Sinn (1956) contain very full and useful surveys.

The original concept (Frank, 1899) was that the arteries functioned like a single elastic reservoir, with pressure-changes occurring simultaneously in all regions, into which blood was pumped by the heart, and from which it escaped into the veins through the resistance afforded by the smaller arteries and capillaries. The "storage" was originally restricted to the central "elastic" arteries, while the peripheral vessels were conceived of as conduits only. This system was considered to be excited anew by each beat of the heart, that is to say that the response to one cardiac impulse had died away completely before the occurrence of the next. The pressure response to the injection of blood by the ventricle was assumed to be due to (1) the elastic behaviour of the Windkessel, and (2) to the properties of the peripheral resistance. It will be seen that this model involves the assumptions that (*a*) the wave-velocity in the elastic reservoir is infinite, so that any pressure-changes occur simultaneously throughout it, and (*b*) that the fluid has no mass. These assumptions are very severe, and a second model has also been set up (Frank, 1905) which takes account of the mass of the system, and the damping within the Windkessel, by likening it to a manometer. Here again the transmission of pressure-changes through the system

was assumed to be instantaneous. The main concept that arose from consideration of this model was that the arterial system possessed a "natural frequency."

As MACEY (1955) has pointed out the most frequent objection to the Windkessel theory has been the assumption of infinite pulse-wave velocity. The introduction of a finite wave-velocity has been attempted in many different ways. In some the initial disturbance has been regarded as instantaneous throughout the system, while subsequent waves were treated as travelling; but this is obviously an unsatisfactory compromise. Another view is that the initial disturbance travels

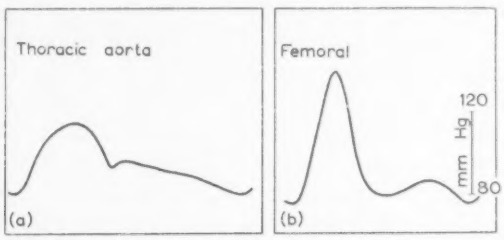

Fig. 1. (*a*) A central aortic pressure pulse; (*b*) A typical femoral pressure pulse (from another animal). Note the striking change in form characterized by a more steeply rising wave-front and increase in amplitude in the femoral pulse together with the development of the dicrotic wave.

through the system, but that all subsequent waves are "standing" and therefore occur simultaneously in all places. The most detailed use of this second concept is that by REMINGTON and HAMILTON (1945, 1947), who have allowed for the varying wave-transmission in the more peripheral parts of the Windkessel, and have also emphasized the importance of standing waves in determining the form of the arterial pulse (HAMILTON and DOW, 1939). This idea of the generation of "standing waves" is linked with that of the "natural frequency" derived from a consideration of the manometer model, but is described in terms of "resonance," dependent on the repeated reflexion of pressure-waves at the terminations of the Windkessel.

1.1. *The explanation of the pulse-form.* Some of the simpler phenomena which these theories were attempting to explain can be illustrated by the comparison of an arterial pulse recorded close to the heart, with one recorded in the femoral artery (Fig. 1*a* and *b*). In the central aortic pulse, there is a marked rise in pressure during ventricular systole, although it has begun to decline before cardiac ejection ceases. This point is marked by the incisura, the notch in the wave which is synchronous with closure of the aortic valves. Thereafter the pressure falls in what is commonly described as an approximately exponential curve, although it may have very minor oscillations superimposed on it. The pulse recorded in the femoral artery, however, has a very different

shape. Not only has the initial wave become more peaked, but a second pressure-wave has appeared which is called the dicrotic wave. This dicrotic wave is a feature of the pulse recorded in all peripheral arteries, and may even be seen in the abdominal aorta. In terms of the manometer model, it has been said that the femoral pulse is like a central arterial pulse recorded with a bad manometer, that is, the higher frequency components, as indicated by the incisura, have been damped out, while it is exaggerating the lower-frequency terms. This has been discussed by BROEMSER (1940); ANDERSSON and PORJÉ (1946) on the basis of his theory, have attempted to evaluate the constants (mass, elasticity and damping) of a manometer that would thus transform a central aortic to a femoral pulse-wave. They found that it was not possible to explain the transformation solely on this basis.

When the shape of the femoral pulse has been explained in terms of travelling waves, the presence of the dicrotic wave is assumed to be due to wave-reflexion, and the interval between the two peaks has been taken as a measure of the "resonant frequency." Together with the measured value of the pulse-wave velocity, this has allowed the estimation of the length of the Windkessel (WEZLER and BÖGER, 1939; WETTERER, 1956; SINN, 1956). A further problem then arises, as to whether the termination of the Windkessel should be regarded as being "open" or "closed." If the cardiac end of the system be regarded as closed, then resonance will occur over a quarter-wavelength if the periphery be open, and over a half-wavelength if it be shut. If the termination is taken as "open" then measurements place it between the bifurcation of the aorta and the inguinal ligament, while if it is taken to be closed, then it is placed more peripherally, in the distal part of the leg. Advocates of the "open-end" type of reflexion base their argument on histological evidence which suggests the elastic type of artery changes into the muscular type at the exit from the abdomen (BENNINGHOFF, 1930), and regard the Windkessel as being coextensive with the elastic arteries. The supporters of the "closed-end" (e.g. WETTERER, 1956; FRANK, 1905) justify their view by evidence for the existence of a "node of pressure" in the region of the abdominal aorta, such as that described by HAMILTON and DOW (1939). From the study of the behaviour of the harmonic components of the pulse-wave, which we describe in detail in Section IV, it would appear that the pulse-wave is reflected from a "closed-end," and that this is situated in the periphery. The arguments leading to this view from deductions as to the length of the Windkessel are suspect for various reasons. As will be seen in the next sub-section, it seems very unlikely that any true resonance, leading to the formation of a node, exists, and the evidence for such a pressure-node is far from being generally accepted. Furthermore, if such a node existed, it would correspond to

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an anti-node of flow, and no evidence for this can be seen in recording of phasic flow along the aorta, as made by SPENCER and DENISON (1956).

2. *The arterial pulse as a transient phenomenon*

An important basic assumption of all Windkessel theories, but one that is rarely stated explicitly, is that each cardiac impulse is an isolated event; therefore, in usual physical terminology, the arterial pulse is treated as a transient phenomenon. A study of the pressure-pulses illustrated in Fig. 1 shows that the main pressure oscillations occur in the earlier part of the cycle, while towards the end of the cycle there is merely a slow decline of pressure. It is this observation which led to the conclusion that the system was then at rest; this can only be regarded as a rough approximation, since inspection of a record of flow in a peripheral artery shows that there are still marked oscillations of flow during this phase of the cycle (Fig. 6).

The analysis of the transient responses of a system has been greatly developed in recent decades with the growth of electrical line theory and practice (e.g. GOLDMAN, 1949). Transient analysis in the technical sense has not, however, been applied to the behaviour of the circulation. To determine the properties of a system from its transient response, one requires first an excitation of known form. This is generally, in electrical work, a step or impulse function, or some other simple pulse-form. The characteristics of the system are then determined by finding the transfer-function relating the Fourier transforms of the excitation and the response. This requires the expression of these functions as Fourier integrals. FRANK (1927) himself indicated that such an approach could be used in the study of arterial pressure responses, but neither he nor his successors employed it. The prime difficulty in its application is the lack of knowledge of the form of the systolic ejection; that is, the driving function is unknown, although it is perhaps not too much to hope that in the future adequate flow-meters applied to the ascending aorta may enable it to be measured. The reverse situation appears in most Windkessel analyses, where, by making assumptions concerning the properties of the system, one attempts to derive the form of the excitation, i.e. the systolic output of the heart, and hence the cardiac output. In fact, all derivations have concerned themselves with the integrated systolic flow, since the estimation of cardiac output is of great clinical importance. However a critical comparison of the estimates arrived at by the use of different Windkessel formulae with those obtained by more direct methods (Fick principle, and dye-dilution) has shown that they are extremely inaccurate (BROTMACHER, 1957), which does not lend strength to the assumptions upon which they are based.

2.1. *The transient response of a simple model.* It may be helpful at this juncture to consider the behaviour of the pressure-changes generated by the sudden injection of fluid into a length of rubber tube (Fig. 2). This model illustrates the main features of the transient response of a system with a finite rate of propagation of disturbances. During the flow into the pipe at the origin, the pressure rises to a value

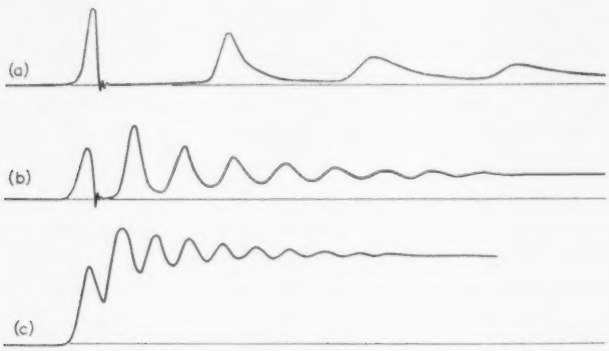

Fig. 2. (*a*) The behaviour of a pressure-pulse due to a sudden injection of fluid, travelling in a long pipe; note the return to base-line pressure until the first reflexion arrives, the progressive spreading of the pulse, and the stepwise approach to the final pressure level. (*b*) Reflexions in a shorter pipe; again the pressure returns to the base-level before the first reflexion arrives, and the more rapid succession of reflexions gives the appearance of oscillations. (*c*) In this example the reflexion arrives during the initial impulse.

which depends upon the "input impedance" of the tube, and is given approximately by the water-hammer formula

$$P = \rho \,.\, \bar{V} \,.\, c \qquad \ldots\ldots(3)$$

where ρ is the density of the fluid, $\bar{V}$ is the linear rate of flow, and c is the propagation velocity. When flow ceases, the pressure falls at once to zero, and remains there until a reflected wave returns from the termination. Until this happens, an observer at the origin has no knowledge of the nature or location of the termination, or indeed whether it exists at all. If the rate of travel of the disturbance be known, then the time taken for the pulse to return gives a measure of the distance of the reflecting site from the origin. If the returning pulse is upright, then reflexion has occurred at a "closed" termination, while if the pulse returns inverted, then it has been reflected from an "open" termination. If it does not return at all, then either the termination is "matched," and gives rise to no reflexion, or the pipe is of infinite extent, or rather of such a length that attenuation has made the returning pulse undetectable.

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The returning pulse differs in form from that first seen, being now blunted before and drawn-out behind. This change in form is due to "dispersion" in travel, since the different frequency components of the pulse-form travel at different rates and are attenuated to different degrees; this dispersion is due to the viscosity, either of the fluid, or in some cases, of the wall-material. It is thus not sufficient to describe the rate of travel of any one particular feature of the wave, for example, the "foot," or the point of maximum pressure; these will all travel at different speeds, and it is best in such a case therefore to use the concept of "group velocity" which measures the rate of propagation of the total energy of the disturbance. After the first echo, one sees that the pressure declines to a new asymptotic level, somewhat above the original level; with successive returns of the echo, this level rises, as it were by steps, until the disturbances have died away. The final level represents then the pressure–volume response of the whole tube. This is only so, of course, if the termination is completely closed. It is not until final equilibrium has been attained that a pressure record can give this information. In the case shown, the duration of the excitation was short in comparison with the "round-trip time" of the reflexion, and so the original impulse and its reflexion remained distinct.

In the circulation, reflexions may return to the origin from a number of sites, arriving there during cardiac systole, and so modifying the shape of the aortic pulse-wave. Fig. 2*c* illustrates the pressure changes in a tube similar to that used for Fig. 2*a*, but of such a length that the first reflexion occurs during the excitation. The initial part of the response until the reflexion returns is, however, still determined by the properties of the tube and not by its termination. The rapid succession of reflexions gives the appearance of an oscillating system, but as SCHMITT (1943) observed, it is not until after the return of the disturbance from the reflecting point that one can speak of a "resonant oscillation." Even then, the influence of attenuation in the system will not allow the production of true standing waves (HARDUNG, 1952; KARREMAN, 1952; WETTERER, 1956; TAYLOR, 1957a).

2.2. *The timing of discrete reflexions in the arterial tree.* Applying these considerations in simple fashion to the cardiovascular system of the dog, let us take a case where the pulse frequency is 2 c/s. Thus out of a cardiac cycle of length 0·5 sec we can assume cardiac systole is 0·2 sec. The mean pulse velocity in the larger arteries is about 5 m/sec, and the length of the aorta about 50 cm. In terms of the models discussed above, this may be regarded as the length of an open-ended Windkessel, and constitutes a quarter-wavelength of the "resonant oscillation". If the Windkessel is regarded as a half-wavelength its closed termination will, of course, be 100 cm from the heart. It will thus be seen that the first reflexion to arrive back at the root of the

aorta will take 0·2 sec to make the round-trip of the shorter Windkessel, and 0·4 sec for the longer. As the primary postulate of the theory is that all waves are completely damped out by the end of the cardiac cycle, i.e. in 0·5 sec, it can be seen that even in the shorter Windkessel, only two round-trips of the system are possible, and the second one must be highly damped if it is to disappear completely 0·1 sec later. It is clear that in these circumstances to talk of the system being in resonance is to use the word in an idiosyncratic manner. In the same way, this condition is not compatible with the existence of standing waves in the accepted physical sense of the word. Admittedly the terminations of parts of the arterial tree such as those supplying the head, the fore-limbs and the viscera, are closer to the heart, and could supply earlier reflexions because of the shorter, segmental round-trip times. However, the bulk of work on the Windkessel has dealt with its extent towards the hind-part of the body, and the problem of transit times forms an objection to the assumptions required for treating the arterial pulse as a transient phenomenon of short duration, which seems to be insurmountable.

2.3. *Experimental use of transient excitation in the aorta.* The logical way to obtain information on the arterial system by studying its transient response appears, then, to be by generating such a response by an excitation of known form, since it is difficult or impossible to measure precisely the excitation provided by the heart. This has been done in recent years independently by STARR and PETERSON. STARR has used human cadavers, from which the heart has been removed, and a syringe substituted. The plunger has been driven in by a blow from a heavy pendulum. In his long series of papers, STARR has been concerned with the calibration of the ballistocardigraph, in the course of which he has made some important observations on the validity of formulae derived from Windkessel theory. The equations tested (STARR and SCHILD, 1957) were those of WEZLER and BÖGER (1939) and of BROEMSER and RANKE (1930). They concluded that the best correlation between the true injection volume and that calculated was achieved after removing the items representing the dimensions of the Windkessel chamber; when this had been done the relation was between stroke volume, pulse pressure and pulse-wave velocity, and was essentially the same as that given by the water-hammer equation (3). They conclude: "so the attempt to define the dimensions of the compression chamber has gained nothing, indeed both methods have lost by it. Therefore our data do not support the compression-chamber theory on which these methods were based." This is particularly significant, because they used a completely isolated injection, which as we have seen is a prerequisite of the Windkessel theory, and the full course of the response could be studied.

Using living animals, PETERSON (1952, 1954) has developed a different technique, in which a rectangular fluid flow pulse can be injected into the aorta, close to the heart, at any pre-determined phase of the cardiac cycle. The pulse he used was of the same volume (20 ml) and duration (0·2 sec) as an average cardiac ejection in the dog; the rise-time to constant flow velocity was 10 msec. A typical pressure-response found by PETERSON shows a sharp step-like rise, followed by a gradual and virtually linear rise until the end of the injection, when there is a sudden drop, followed by a more gradual decline. Assuming once more that the pulse-wave velocity is 5 m/sec it can be seen that during the 10 msec required to attain constant velocity of flow, only the initial 5 cm of the aorta can have been involved in the disturbance. The magnitude of the step in pressure must have been determined by the local pulse-wave velocity dependent on the dimensions and elastic properties of the aorta. The steady rise thereafter we visualize as being similar to the condition illustrated in Fig. 2*c*, where successive reflexions are superimposed on the initial response. As no discrete waves are seen in his records it seems clear that reflexions are more diffuse than in the rubber tube, which is almost certainly due to the fact that they are arriving from a series of reflecting sites at different distances from the heart. If discrete reflected waves were to be found one would also expect them to be set up following the drop in pressure at the end of the injection. The absence of such clear-cut oscillations is also to be seen in the tracings reproduced by STARR (1957). Such experimental evidence as there is, therefore, does not support the assumption that any portion of the pressure-pulse curve can be explained as a reflexion phenomenon from any one point, or that reflected waves can travel over the system from the periphery to the heart and back again, and retain a discrete identity. STARR and SCHILD's work emphasizes once again, as we saw in the rubber-tube model, that in terms of a transient response, the initial rise in pressure in an aortic pulse-tracing must be determined solely by the local elastic properties; but without a knowledge of the form of systolic ejection, no quantitative use can safely be made of this.

3. *The arterial pulse as a steady-state oscillation*

Perhaps the most striking feature of the pulse is its regularity. In terms of the duration of the cardiac cycle this regular rate, in the normal animal, is maintained for long periods. It therefore seems more realistic to regard the arterial pulse as a compound wave with a fundamental frequency that of the heart-rate. The arterial circulation will thus be regarded as being in a steady state of oscillation. This emphasis on the repetitive nature of the pulse constitutes the only fundamental assumption of the investigations that are presented in the following

sections. This is in marked contrast to the Windkessel theories, which as we have seen require very restrictive simplifications and which are thereby limited in the extent to which they may adequately describe circulatory events. Furthermore, many of the assumptions which have been employed are physically unfounded, or mutually incompatible.

The physical analysis of steady-state phenomena is by their representation as Fourier series (equation 4) whereby a repetitive pulse-form is expressed as a set of continuous sinusoidal oscillations. A system is said to be in a steady state once any disturbances due to the starting up of the motion have died away; in practical terms we have found that the arterial system is highly damped, and that it reaches a steady state very rapidly after an alteration in heart-frequency has occurred, certainly within three to four beats. Thus although small variations do occur in the length of the cardiac cycle even under stable physiological conditions, the experimental approximation involved in well-chosen records is negligibly small. Under these conditions the Fourier representation is a perfectly valid description of pressure *or* flow events, but to relate pressures *and* flows requires that the equations of the system be linear. That is to say, that whereas in the series the individual harmonic terms are independent of each other and are simply additive, we make the assumption that one harmonic term of the pressure-pulse is related only to the corresponding harmonic term of the flow-pulse. This assumption involves a much larger approximation than the first one, because the arterial system is undoubtedly non-linear in most of its properties. The non-linearity arising from the elastic expansion of the wall and the interaction of inertia terms in oscillatory flow has been estimated by Womersley (1955a, 1958a), and has proved to be considerably smaller in magnitude than the experimental errors. The effects of the non-linear elastic properties of the arterial wall have not yet been fully investigated, but for small variations about a mean pressure the error involved in assuming linear behaviour is probably not prohibitive. In the present state of the experimental techniques involved, the errors due to such an assumption of linearity are obscured by the great difficulties of precise measurement. With improvement in methods, the effects of non-linearity may become manifest, and need to be taken into account. Further approximations are made in the course of the analysis, for example, decisions as to the number of terms in the series necessary to give sufficient accuracy of description of the pressure or flow curves; but none involve any fundamental principles, and the magnitude of such approximations can always be estimated.

The idea of using the Fourier series to represent the pulse-wave has been put forward by various workers, notably Frank (1905), Broemser

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(1930), MATOBA and KAJIURA (1933) and APÉRIA (1940). While FRANK was fully aware of its potentialities as an analytical method, as witness his later theoretical paper (FRANK, 1927), he never exploited it. BROEMSER (1930) applied the method fully in studying the responses of recording instruments and also (BROEMSER, 1940) in attempting to determine the frequency responses of the arterial system. MATOBA and KAJIURA only used Fourier series to describe the shape of pulse-waves under varying conditions. The implications, however, of treating the harmonic series as a representation of a steady-state oscillation were only developed by PORJÉ (1946) taking up the suggestions of APÉRIA (1940) in a study of the phase velocities of the harmonic components of the pulse-wave which ascribed their differences to wave-reflexion. In using theoretical models the assumptions of steady-state have also been taken by LANDES (1949), RONNIGER (1954) and KUGA (1954), but have only been applied to the physiological situation to a limited extent. In our work the assumption of steady-state oscillations is fundamental.

3.1. *Main topics of investigation.* The first aspect of the physical properties of the circulation that was investigated in this department was the relation of the pressure-gradient to the flow in a single artery and is considered fully in Section II. The agreement between the experimental results and those predicted by the pressure-flow relations of a single sinusoidal component, was reasonably satisfactory. The theory applied was of a simple form which neglected arterial expansion, and attempts to improve on this have led to a detailed analysis of the hydrodynamics of oscillatory fluid motion in a free elastic tube, and also in elastic tubes which are constrained in various ways, as by the connective tissue attachments of arteries in the body. The consideration of the relations between the motion of the liquid and the pressure-gradient led to the study of the relations between the pressure-gradient and the pressure-wave-form. This latter relation is simple, but requires a knowledge of the apparent phase velocity of each of the harmonic components of the wave; distortion of the wave-form as it travels (Fig. 1*a*, *b*) indicates that this can be very different at different frequencies.

We have seen that in a system of branching elastic tubes, such as the arterial tree, wave-reflexion must occur, and there is general agreement that this is an important factor in the distortion of the wave-form. Special consideration of this topic is given in Section IV.

We have already considered the behaviour of a single impulse travelling and being reflected, when it is possible to distinguish the centrifugal wave from the centripetal reflected wave. If, however, the impulse is repeated at regular intervals, we create as we have seen above, a steady-state oscillation. The centrifugal and centripetal harmonic components now fuse to give rise to a set of harmonic waves which are

their resultants. The limiting form, in a system with no damping and complete reflexion, would be a set of stationary, or "standing", waves. Thus the basic assumption which regards the regular rhythm of the heart as creating a steady-state of oscillation in the arterial tree precludes any attempt at analysing pressure curves by inspection, to distinguish "incident" and "reflected" wave-components. The estimation of the reflected components of the composite wave can only be made by harmonic analysis of the wave-form at successive points along the arteries. In the presence of damping, even where there is complete reflexion, no true standing wave can be established, although points of maximum and minimum pressure variation will occur, which may be referred to as "relative" anti-nodes and nodes. In addition to variation in amplitude from point to point, there will also be variation in the phase of an oscillation; the rate of this phase-shift per unit length of vessel may be expressed as an *apparent phase velocity*. This concept is unfamiliar in physiology, but is useful in detecting the presence of reflexions and in estimating their magnitude.

Another conclusion that derives from the analysis of the steady-state oscillation is that the concept of "natural frequencies," such as have been discussed in relation to the Windkessel models, must be considerably modified. This concept was drawn by analogy with a system, such as an organ-pipe, which when excited by a wide range of frequencies, will resonate and hence amplify the fundamental frequency, and its overtones, that are appropriate to its length. This analogy was never a good one for the arterial system, in which damping is high and in which complete wave-reflexion does not occur, but it did represent a physical possibility. When, however, it is appreciated that the heart is driving the arterial system at a constant frequency, we have to consider a system which is in a condition of forced oscillation. Here, the only frequencies that can appear are integral multiples of the fundamental, i.e. the pulse, frequency. The pressure response to an oscillatory component of the cardiac ejection pattern will depend on the relation of its frequency to a possible resonant frequency of the arterial system. This indicates the experimental means whereby the frequency-responses of the arterial system could be determined. By imposing flow-oscillations of different frequencies and measuring the resulting pressures, the existence of any resonant range of frequencies could be found (McDonald and Taylor, 1956).

3.2. *The physical characteristics of the arterial tree.* In all systems involving wave transmission of known frequency, the wave-length is a fundamental unit. Many of the peculiar properties of the pulse-wave in the arterial system are due to the fact that the whole system is only a fraction of the fundamental wave-length. Referring to the "average," or arithmetically simplified, dog already discussed, in which

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the wave-velocity is 500 cm/sec and the pulse frequency 2 c/s, we see that the wave-length of the fundamental oscillation is 250 cm. Compared with this, the aorta is about 50 cm in length, and the whole system at the most distant arterioles not more than 80–100 cm from the heart. The wave-length of the second harmonic is still 125 cm and at least 75–80 per cent of the energy of the pulse-wave is represented by these two components. Only in the fourth and higher harmonics, which are all small in magnitude, does the system significantly exceed one wave-length. This consideration of dimensions obviously limits the number of relative nodes and antinodes which can be created in the system.

The calibre of the vessels which we are considering is, in terms of wave-length, very narrow. Even in conventional units they are small compared with those most commonly considered in hydraulics, so that in spite of the fact that blood is a liquid of low viscosity (hydrodynamically speaking) the Reynolds numbers found in most arteries are fairly low, and the flow may be regarded as laminar. This is only an approximation in the large arteries but, for reasons discussed below, it is one which on present evidence does not appear to introduce any major error.

The static elastic behaviour of the arterial wall is markedly non-linear in its stress–strain relationship although precise information on this is surprisingly lacking. This has been reviewed by Burton (1954) and McDonald (1958). The effect of this non-linear behaviour on the velocity of pulse propagation is discussed in Section III, in relation to changes in arterial pressure. An oscillatory pressure disturbance in such a system will be distorted by the generation of higher-frequency terms, but provided that the amplitude of the wave is small they may, as a first approximation, be neglected. A distinction must also be made between this effect and the visco-elastic properties of the wall which account for the change in dynamic elastic behaviour. The latter will depend on the frequency of the disturbance, while the former depends on its amplitude.

There are very few records of the dynamic behaviour of the arterial wall. Hardung (1953) has measured the stress–strain characteristics of strips of rubber and of aortic wall, over the range of frequencies 0–15 c/s, and with varying initial extensions. He has computed from these measurements the dynamic elastic modulus in the form

$$E_{\mathrm{dyn}} = \frac{\Delta P}{\Delta l} \cdot \frac{l_m}{q_m} \cdot \cos\phi$$

$$\eta\omega = \frac{\Delta P}{\Delta l} \cdot \frac{l_m}{q_m} \cdot \sin\phi$$

where the complex elastic modulus is thus $E' = E_{\mathrm{dyn}} + i\eta\omega$. ΔP and Δl are amplitudes of the stress and strain respectively, l_m and q_m are

the average values of the length and cross-section of the specimen, while ϕ is the phase difference between force and extension; $\omega = 2\pi f$ and η is the viscosity-coefficient. The results he presented show that in general E_{dyn} was greater than E determined statically, and did not change greatly with frequency, whereas the term $\eta\omega$ increased linearly. HARDUNG was not concerned in this paper with the calculation of the frequency-dependence of wave-velocity in tubes composed of these substances, but clearly the effects which he demonstrated must be taken into account in such calculations. LAWTON (1955) has also measured the dynamic elastic modulus of strips of thoracic aorta of the dog. His values were plotted against the extension imposed on his strips, but their frequency-dependence is not defined. REMINGTON (1955) has investigated the behaviour of rings of arterial wall in response to single stretches of varying rapidity. This grew out of the observation that pulse-wave velocity (foot-to-foot) did not correlate well with the elastic behaviour of rings stretched under static conditions (HAMILTON *et al.*, 1945).

While technical simplicity recommends the study of strips of arterial wall, whether longitudinal (LAWTON) or circumferential (REMINGTON), it must be noted that predictions about the behaviour of the whole artery from such strips necessitates additional assumptions of isotropism and the absence of interaction between elastic fibres orientated longitudinally and circumferentially. Although these assumptions may be reasonable, it would be better to avoid them, as can be done by using intact arterial specimens. As the length of arteries appears to be nearly fixed in the body, it is suggested that the best arrangement for their study is a segment of artery which is pulled out and held at its natural length, and then subjected to a sinusoidal pressure oscillation of known frequency.

An apparatus of this kind has been developed by BERGEL (1958), in which the diameter of the specimen is measured photoelectrically; no data on arteries are yet available. Preliminary experiments on rubber tubes give similar results to those of HARDUNG. If it proves possible, by the substitution of the complex elastic modulus in an expression such as the Moens–Korteweg formula (equation 2), to obtain values for the complex wave-velocity which agree with those found directly in long segments of such tubes, the same method could be used to determine the frequency-dependent wave-velocity of oscillations in an artery.

II. THE PHYSICAL LAWS GOVERNING THE FLOW OF BLOOD

The biological function of the heart is to pump blood to the tissues of the body. For any study of the circulation the consideration of the

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laws governing the motion of the blood is of fundamental physiological concern. Equally a knowledge of the pressure-flow relationship in a system is of primary importance in any hydrodynamic investigation. Any account of the circulation that does not cover this topic is necessarily incomplete. The preoccupation of the Windkessel theory with the behaviour of pressure-waves in arteries is one of its major defects.

The heart creates a regular, intermittent ejection of blood and so flow in arteries is pulsatile. We therefore choose to describe this flow and the accompanying pressure-waves by Fourier series. Such a series is represented by

$$F(t) = A_0 + \sum_{m=1}^{m=\infty} A_m \cos m\left(\frac{2\pi}{T}\right)t + B_m \sin m\left(\frac{2\pi}{T}\right)t \quad \ldots . (4)$$

(where T = cycle-length) so that the pulsatile flow consists of a set of terms which oscillate around a mean value A_0. This mean value we refer to as the steady flow and the remainder as the oscillatory flow. Similarly the pulse-pressure curve is described as a set of pressure-waves oscillating about a steady mean pressure.

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Poiseuille determined the pressure-flow relation for steady laminar flow over a century ago and the full mathematical expression of this (equation 1) is almost a hundred years old. Since then a considerable body of detailed work as to the applicability of Poiseuille's law, most especially in the smallest vessels of the circulation, has been published. The pressure-flow relations of the oscillatory component, however, have been almost completely neglected. We shall, therefore, first consider this aspect of flow.

1. *The flow related to an oscillating pressure-gradient*

It is clear that if the pulsatile flow and pressure in arteries are expressed as a Fourier series then the elementary case to consider is that of simple harmonic motion of liquid in a cylindrical tube. The mathematical treatment is initially similar to the standard derivation of Poiseuille's law (Lamb, 1932; Newman and Searle, 1948). The basic assumptions made are the same, with the single difference that whereas in steady flow the pressure-difference, $P_1 - P_2$ between the two ends of the pipe of length L is constant with time, in the present case the pressure-gradient $(P_1 - P_2)/L$ oscillates in harmonic motion. The other assumptions on which the analysis is based are repeated here, although they are to be found in any standard textbook, because it will be necessary to consider them in discussing the validity of the result when applied to the circulatory system. These conditions are:

1. The flow is laminar.
2. The tube is long, i.e. we have established flow beyond the entrance, or inlet length.

3. The viscosity of the liquid is independent of the rate of shear, i.e. it is a Newtonian liquid.
4. There is no "slip" at the wall.
5. The radius of the tube does not vary, e.g. with changes of pressure.

The form of the solution given here follows that of WOMERSLEY (1955a, b). The equation of motion is a unidimensional form of the Navier–Stokes equation and can be written

$$\frac{\partial^2 w}{\partial r^2} + \frac{1}{r} \cdot \frac{\partial w}{\partial r} + \frac{P_1 - P_2}{\mu L} = \frac{1}{\nu} \cdot \frac{\partial w}{\partial t} \qquad \ldots.(5)$$

where w is the velocity of the liquid parallel to the axis of the pipe (the z-axis) at a distance r from the axis. The viscosity of the liquid is μ and the kinematic viscosity ν (μ/ρ, where ρ is its density). The radius of the pipe is R and r may be written as a fraction $y = r/R$.

If the flow is steady, i.e. does not vary with time, then the right-hand side of equation (4) equals zero and the solution required is

$$w = \frac{P_1 - P_2}{4\,\mu L} \cdot R^2(1 - y^2) \qquad \ldots.(6)$$

In the present case we take a pressure-gradient which is periodic with time with a circular frequency ω so that

$$(P_1 - P_2)/L = -A\,\mathrm{e}^{i\omega t} \qquad \ldots.(7)$$

and the equation of motion is rewritten

$$\frac{\partial^2 w}{\partial r^2} + \frac{1}{r} \cdot \frac{\partial w}{\partial r} - \frac{1}{\nu} \cdot \frac{\partial w}{\partial t} = \frac{A}{\mu}\,\mathrm{e}^{i\omega t} \qquad \ldots.(8)$$

Let $w = u\,\mathrm{e}^{i\omega t}$ and let the non-dimensional quantity $R\sqrt{(\omega/\nu)}$ be denoted by α. Then the equation for u is

$$\frac{\partial^2 u}{\partial y^2} + \frac{1}{y} \cdot \frac{\partial u}{\partial y} + i^3\alpha^2 u = \frac{AR^2}{\mu} \qquad \ldots.(9)$$

which is a form of Bessel's equation and the required solution after replacing w is

$$w = \frac{AR^2}{\mu} \cdot \frac{1}{i^3\alpha^2}\left\{1 - \frac{\mathrm{J}_0(\alpha y i^{3/2})}{\mathrm{J}_0(\alpha i^{3/2})}\right\}\mathrm{e}^{i\omega t} \qquad \ldots.(10)$$

where J_0 is the Bessel function of the first kind and of zero order.

This solution has been derived by a number of workers. WITZIG (1914) appears to have derived it although we have not been able to get access to his thesis. It was used in studying oscillatory airflows by RICHARDSON and TYLER (1929), which work was further discussed by SEXL (1930). SCHÖNFELD (1948) published this solution and that for

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other forms of channels and applied them to a study of tidal flows in canals around the Zuider Zee. LAMBOSSY (1952) gave the solution in the form of equation (10) writing z for the non-dimensional parameter α that WOMERSLEY used. He was primarily concerned with manometers, but calculated the resultant velocity profiles and these were found to agree with experimental determinations by MÜLLER (1954). WOMERSLEY's solution was derived independently and has been taken further in that he also integrated equation (10) to give the solution for the volume flow which is necessary for application to the arterial system

$$Q = \frac{\pi R^4}{\mu} \cdot \frac{A}{i^3\alpha^2}\left\{1 - \frac{2J_1(\alpha i^{3/2})}{\alpha i^{3/2} J_0(\alpha i^{3/2})}\right\} e^{i\omega t} \qquad \ldots.(11)$$

These equations (10) and (11) may be computed by separation into real and imaginary parts in terms of *ber* and *bei* functions. This is the method used by LAMBOSSY (1952) and has been greatly facilitated by the tabulation of the bracketed term in equation (11) by WOMERSLEY (1958a). Originally, WOMERSLEY (1955b) computed them by expressing the results in modulus and phase form, and as the physical behaviour of oscillating flow is thereby more easily appreciated we will use this nomenclature.

If the real part of the pressure-gradient $A\,e^{i\omega t}$ is $M\cos(\omega t - \phi)$ then the equation for volume flow is written

$$Q = \frac{\pi R^4}{\mu} \cdot \frac{M \,.\, M'_{10}}{\alpha^2} \sin(\omega t - \phi + \varepsilon_{10}) \qquad \ldots.(12)$$

M'_{10}/α^2 and ε_{10} are functions of α. They are defined and tabulated by WOMERSLEY (1955b) for functions of $\alpha = 0(0{\cdot}05)10{\cdot}0$. Fig. 3 is plotted from the same table.

1.1. *The significance of the parameter α in determining oscillatory flow.* If the equation 12 is compared with that of POISEUILLE's formula (equation 1), remembering that the pressure-gradient $(P_1 - P_2)/L$ is written as $M\cos(\omega t - \phi)$ in equation 12, the similarity can be seen at once. The factor M'_{10}/α^2 modifies the modulus, or amplitude, of the flow and, in addition, a phase shift ε_{10} is introduced, so that flow lags $(90 - \varepsilon_{10})^\circ$ behind the pressure-gradient. From the tables of WOMERSLEY (1955b), which are plotted graphically in Fig. 3, we find that as $\alpha \to 0$, $M'_{10}/\alpha^2 \to 1/8$ and $\varepsilon_{10} \to 90^\circ$. The formula then becomes identical with that of POISEUILLE. On the other hand as $\alpha \to \infty$, $M'_{10}/\alpha^2 \to 1/\alpha^2$ and $\varepsilon_{10} \to 0$.

Thus, for a given pipe, if the pressure-gradient oscillates at a very low frequency so that α is small, then the flow will oscillate with it with a negligible phase lag and its amplitude will be nearly that given by POISEUILLE's formula for steady flow. As the frequency increases

the amplitude of the oscillation of the fluid, with a constant pressure oscillation, will diminish progressively until it is imperceptible and the phase lag will approach 90°. Considering the inertia of the liquid, it is easy to appreciate the effect of increasing the frequency of the impressed oscillation. It is not so immediately obvious that changes in the calibre of the pipe should have a similar effect. The value of α,

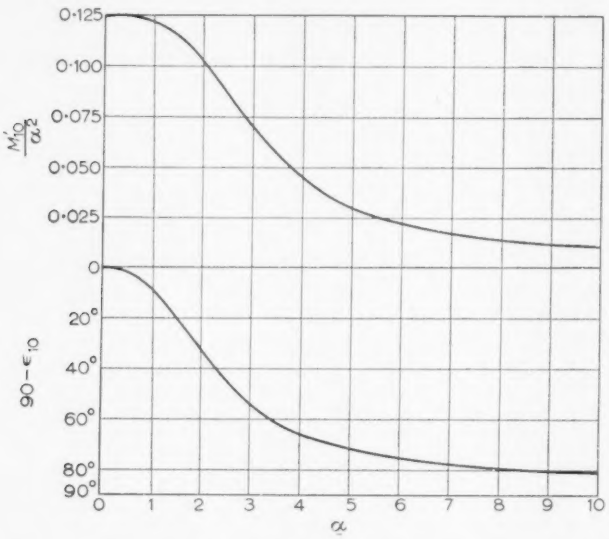

Fig. 3. The quantities M_{10}'/α^2 and $(90 - \varepsilon)°$ plotted as functions of α. It is to be noted that at $\alpha = 0$, $M_{10}'/\alpha^2 = 1/8$, agreeing with Poiseuille's formula, while the phase lag is zero (after WOMERSLEY, 1955b).

it will be recalled, increases linearly with the radius of the pipe and with the square root of the frequency, that is,

$$\alpha = R\sqrt{(\omega/\nu)} \qquad \ldots.(13)$$

1.2. *The values of α in the arterial system.* In the circulation the fundamental frequency is the pulse frequency and is, of course, the same throughout the system. In any species this frequency will not vary by more than a factor of 3, and changes in α due to this will be relatively small, i.e. by a factor of $\sqrt{3}$. The higher harmonics require us to compute a range of values for α in any one artery, e.g. the 4th harmonic will have a value of α twice that of the fundamental and the 9th harmonic an α three times as large. Fortunately, as the amplitude of the flow oscillation diminishes markedly with frequency, the contribution to the total flow of the higher harmonics in the pressure-gradient is small, and we have never found it necessary to calculate

flows beyond the 6th harmonic for its contribution is rarely more than 1 or 2 per cent.

The range of values of α found in the circulation due to the changes in radius are, by comparison, very large. Once more considering a dog with a pulse frequency of 2 c/s the radius of the proximal aorta is about 0·5 cm. The value that we use for the viscosity of the blood is 0·04 poises and its density 1·05 g/ml. The value of α for the fundamental here is thus about 9·0. At the distal end of the aorta, if $R \sim 0{\cdot}3$ cm—$\alpha = 5{\cdot}4$; in the femoral artery $R \sim 0{\cdot}15$ cm—$\alpha = 2{\cdot}7$; in the saphenous artery $R \sim 0{\cdot}05$ cm—$\alpha = 0{\cdot}9$ and in arterioles, where some oscillatory flow persists, $R \sim 0{\cdot}005$ cm—$\alpha = 0{\cdot}09$. The range is thus about an hundredfold difference in magnitude. The values of α for man are somewhat larger but are comparable as the increase in radius is partly offset by the lower pulse frequency normally found.

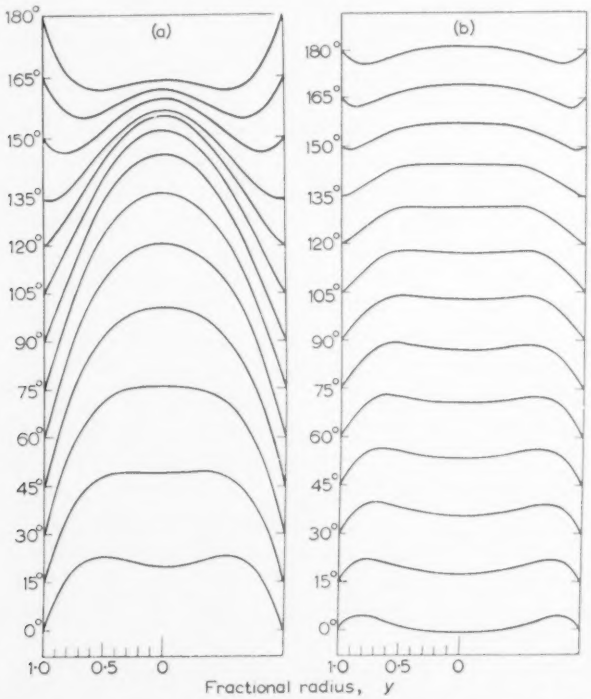

Fig. 4. Velocity profiles at intervals of 15° over a half-cycle for the flow resulting from a sinusoidal pressure-gradient in a pipe. The modulus of the gradient is the same in both cases, but the frequency in (*b*) is four times that in (*a*). Note that reversal of flow starts in the laminae near the wall. (*a*) in this case $\alpha = 3{\cdot}34$; only at maximum excursion does the profile approach a parabola. (*b*) here $\alpha = 6{\cdot}67$; note the marked flattening of the profile in the central region of the tube, and the great reduction in amplitude (from HALE *et al.*, 1955).

1.3. *The effect of changes in radius on the characteristics of flow: the velocity profile.* The fact that the deviation of pressure-flow relations from that of POISEUILLE's formula is marked, even at low frequencies in a pipe of large calibre, can only be fully understood by studying the distribution of flow velocity across the lumen. This "velocity profile" in steady flow is a parabola (equation 6). When the flow becomes oscillatory the profile at maximum flow becomes progressively flatter in the more axial regions of the pipe. LAMBOSSY (1952) was the first to calculate such profiles for values of α in the range we find in arteries (for $\alpha = 2{\cdot}85$ and $\alpha = 7{\cdot}24$). MÜLLER (1954) measured one in a model for $\alpha = 6{\cdot}43$. HALE *et al.* (1955) calculated them for the first four harmonics of the flow-pulse in a dog's femoral artery in which there was good agreement between the observed and calculated average* velocities of flow. The values of α in this example were 3·34, 4·72, 5·78 and 6·67. Some illustrative examples are shown in Fig. 4. They also computed the composite profile, with a parabola added for the steady flow term, for the total flow in the femoral artery. The most interesting point about the oscillating profiles is that the reversal of flow first occurs in the laminae close to the wall and progressively involves the laminae towards the axis. The motion of the juxta-mural laminae is most closely in phase with the pressure-gradient and the phase lag increases towards the axis as shown in Fig. 5. This discrepancy of phase is such that reversal of flow occurs in the curve $y = r/R = 0{\cdot}95$ rather more than 30° in advance of that at the axis, $y = 0$.

In simple conceptual terms this difference in the pattern of the motion of the flow across the pipe may be thought of as the interaction of the dominance of viscous effects close to the wall and inertial effects in the axial regions. Some light might be shed on oscillatory flow patterns by regarding them as a boundary layer problem in hydrodynamics such as discussed by GOLDSTEIN (1938, Chapter 4). Certainly with the larger values of α, the liquid may reasonably be considered as being either in the region adjacent to the wall in which there is shearing, i.e. with a velocity gradient, or in the relatively unsheared axial region. This situation is one in which the criteria by which we define laminar or turbulent flow should be used with caution.

The hydrodynamics of oscillatory motion in liquids has been relatively little explored. The velocity profile with $\alpha > 5$, considered in terms of boundary layer formation, suggests an analogy with the conditions within the inlet length (e.g. GOLDSTEIN, 1938, Fig. 82). In this case the oscillatory flow pattern may be established much closer to the

* *Average* velocity is the average across the pipe and is obtained by dividing the volume-flux by the cross-sectional area, i.e. $Q/\pi R^2 = \overline{V}$. The term *mean* velocity is reserved for the mean value throughout the cardiac cycle.

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origin of the aorta than would be predicted for steady flow conditions (McDonald, 1958). The assumption that the flow in the proximal aorta may be regarded as largely unsheared has been used implicitly

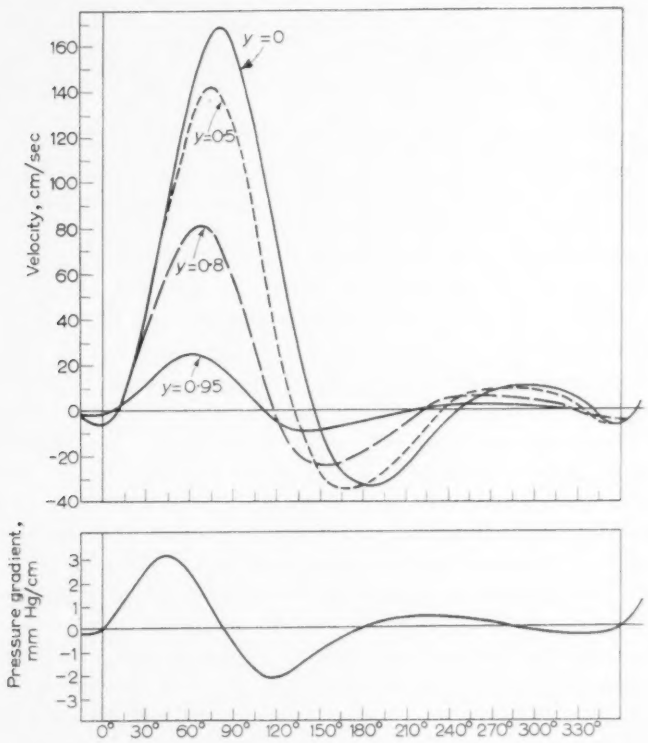

Fig. 5. The velocity of pulsatile flow in the dog femoral artery, displayed in terms of individual laminae in the stream. The position of a lamina is defined by $y = r/R$, thus $y = 0$ at the axis, $y = 0{\cdot}5$ midway between the axis and the wall, and $y = 0{\cdot}95$ close to the wall. The measured pressure-gradient from which the curves were computed is displayed below. It can be seen that the flow of fluid near the wall follows the pressure-gradient most closely, and that the phase lag increases to a maximum at the axis. The peak average forward velocity was 105 cm/sec at 75° and the peak backward velocity was 25 cm/sec at 165° (from Hale *et al.*, 1955).

by Fry *et al.* (1956) in deriving a pressure-flow relation that is simpler than the more general form given by Womersley and discussed above.

The tendency for vortices to originate at the margin of the boundary layer (Goldstein, 1938) may also explain the observation of Helps and McDonald (1954) that large vortex rings were formed when flow reversal took place in the inferior vena cava. These rings did not involve the blood close to the walls of the vein nor the axial regions.

2. *The experimental testing of the mathematical theory*

At the present time there is no experimental evidence that tests the validity of the equation for oscillatory flow which is in any way comparable with the precision with which POISEUILLE established the law for steady flow. Such rigour can only be achieved with hydraulic models and such investigations as have been made are only in a preliminary stage.

2.1. *Tests in physical models.* STACY and POTOR (1958) created oscillatory flows with a pump, which they measured in an electromagnetic flow meter and related these to the pressure-gradient across the meter. They plotted the curves in relation to the values of α as in Fig. 3 and obtained good general agreement. The liquids they used were water, glycerine and blood, and it is of particular interest in the physiological application of the above equations (11, 12) that blood, although a suspension, behaved in the same way as the other two liquids. It is well-known that blood shows anomalous viscous properties in small tubes (L. E. BAYLISS, 1952) but this is very small with steady flow in tubes of radius larger than 0·01 cm. STACY and POTOR used a tube of 0·08 cm radius, that is about half the size of the femoral artery of a dog. This evidence appears to validate the assumption that in all arteries where there is appreciable oscillatory flow the blood may be treated as a homogeneous Newtonian fluid (p. 124 above). It is still desirable, however, that really precise measurements should be made although, in view of the orthodox nature of the derivation, this aspect is not perhaps of primary importance. The experimental scatter in STACY and POTOR's results is much less than the errors inevitable in measurements made in the living animal. As noted above MÜLLER (1954) has also recorded the velocity profile of oscillating flow in a rubber pipe with a Pitot-tube and found good agreement with that predicted by the mathematical theory.

2.2. *Tests in the living animal.* The first tests of the Womersley equation (12) were made on measurements of pressure-gradients and phasic flow velocities in the femoral artery of the dog (MCDONALD, 1955). One of the results comparing a calculated and an observed flow curve is seen in Fig. 6. The agreement between the two curves is reasonable, and results are always of the right order of magnitude but the fit is far from perfect.

The method used for recording phasic flow velocity was that of MCDONALD (1952a) in which bubbles of oxygen are injected into the artery and their rate of travel recorded by high-speed cinematography (*c.* 1500 frames/sec). The results from this technique had shown far greater phasic variations in velocity than those recorded by most other methods, especially electromagnetic flowmeters (this evidence is

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discussed in detail by McDonald, 1958). The variations between the published records of the phasic flow in the dog femoral artery had made it important to establish a criterion against which meter performance could be measured. The bubble method, although theoretically acceptable, is over-elaborate for general use but the check against the calculated flow is available to all. This should be of value in designing flowmeters, for the lack of accurate instruments of this type is a major technical barrier to the progress of circulatory research.

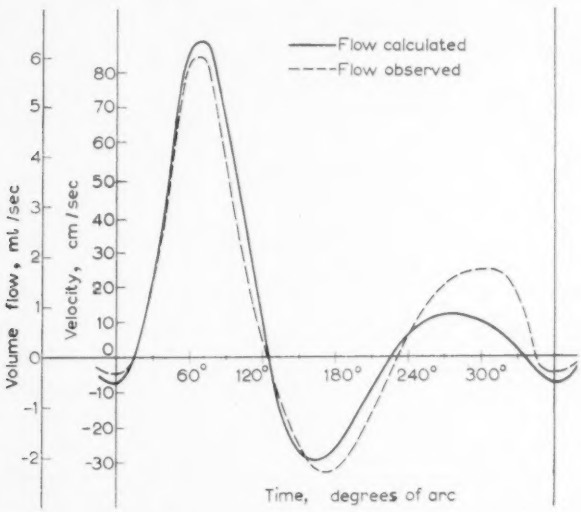

Fig. 6. The velocity of flow measured by high-speed cinematography is compared with a flow calculated from the time-derivative of the pressure measured simultaneously. Although the curves are very similar in form, the discrepancies introduced by the use of the time-derivative are discussed in the text.

The discrepancies between the curves, as in Fig. 6, may be due either to mensuration errors or to deficiencies in the theory, especially in the simplifying assumptions on which it is based. This includes a consideration of the method of deriving the pressure-gradient which in many experiments was not measured directly. Although the technical precision of the pressure measurements, and more especially of the flow recording, was low by physical standards, the fact that the discrepancy was proportionately far greater in the diastolic part of the flow curve than in the systolic part led us to believe that defects in some of the theoretical assumptions were probably present.

The assumption that the elastic behaviour of the artery was of negligible effect seemed the most obvious defect (although the maximum variation in radius of the femoral artery was found to be only about 4 per cent at most). Womersley (1955a) therefore undertook a detailed

study of the motion of liquid in a free thin-walled elastic tube but the modification of the calculated flow pattern was not marked. Later WOMERSLEY (1957) made a further investigation of a more realistic model of an artery, an elastic tube subject to constraints by the surrounding tissues. This led to the conclusion that the behaviour of the wall had little effect on the motion of the liquid, within the limits to which we could measure it, and that the original simple theory was the most satisfactory approximation. These theoretical investigations shed a great deal of light on the physical analysis of other aspects of the arterial circulation which are discussed below, but make it clear that neglecting the behaviour of the wall is not the cause of significant errors in estimating the oscillatory component of the arterial flow.

This conclusion led to a critical consideration of another of the assumptions, namely, that we have a long pipe so that flow is not modified by end-effects. Mention has already been made of alterations in flow-pattern due to effects close to the inlet, but these almost certainly do not apply to the femoral artery. In a system with wave-transmission there is also the possibility of effects due to the proximity of the far end of the pipe which we had neglected, but which we have found to be relevant in deriving the pressure-gradient.

2.3. *The pressure-gradient in an artery.* It has been emphasized throughout the present section that it must be the pressure-*gradient* that governs the flow of the liquid. Although every circulatory physiologist is familiar with this term in POISEUILLE's equation it appears to have been overlooked in investigations of arterial flow. The standard method of measuring a pressure-gradient in a tube is by differencing the pressure recorded at either end of a measured length. With a tube in which the pressure-wave is transmitted with a finite velocity this is not quite so simple because the change in flow-velocity also travels along the pipe. Ideally, to study their hydrodynamic relations the pressure-gradient, and the flow, should be measured at a point. The difference method, therefore, should be applied to as short a length as possible. In practice, when applied to an artery, this must be so if there are to be no intervening branches.

Technically, this introduces considerable difficulties. Even in a favourable situation such as the femoral artery the recording points can rarely be more than 7 cm apart and reference to Fig. 5 shows that the maximum gradient at any point is about 3 mm Hg/cm, i.e. a maximum difference of about 20 mm Hg, while the diastolic peak will only give a difference of about 7 mm Hg. MCDONALD (1955) used capacitance manometers which have a range of 0–200 mm Hg and which the makers claim to be accurate within 2 per cent of that range, i.e. 4 mm Hg. Even ignoring the problem of exactly matching the calibration of two different manometers, it is clear that a gradient subject

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to such an experimental error must be grossly inaccurate. It is, therefore, necessary to use a differential manometer in which the mean pressure in the artery at the two points will be eliminated from the record. Such a manometer has only recently become available to us, so that in the original investigations an indirect method of deriving the pressure-gradient was used, once the experimental variability of the direct method was appreciated.

In measuring the pressure-gradient we are measuring $\Delta P/\Delta z$. The gradient at a point, the limit, is thus $\partial P/\partial z$ the derivative with respect to distance. As the wave-velocity is $\partial z/\partial t$ it can be seen that there is a simple relation between the derivative with respect to time and the gradient

$$\frac{\partial P}{\partial z} = -\frac{1}{c} \cdot \frac{\partial P}{\partial t} \qquad \ldots\ldots(14)$$

(the negative sign is introduced because the slopes are in opposite sense).

This is true in this simple form for a sinusoidal wave, and hence, following the assumption of the linear addition of Fourier terms, for a compound wave provided that the wave is travelling without distortion. In other words, provided that the value c is the same for all the harmonic components. As the whole treatment of the motion of the blood at the time this work was done was based on the concept of a long tube the assumption was made that the value of c to be taken was that of the pulse-wave velocity in the femoral artery. A method for measuring this velocity using a quartz oscillator chronometer was used for recording the wave-transmission time over short distances in individual arteries (Hale *et al.*, 1955). It was appreciated that, owing to the effect of the viscosity of the blood, the wave-velocity would vary with frequency to some extent. Womersley (1955a) had calculated this effect (Fig. 9a) but it can be seen that with values of α greater than 3 this frequency-dependence is slight and it was regarded as negligible. In practice this approximation works admirably in many cases, as shown by Fig. 6 in which the pressure-gradient was derived by this method. In studying a variety of physiological states it was found that the best agreement between theory and observation was found where the arteriolar bed was dilated, and that large discrepancies occurred in cases where there was marked vasoconstriction.

This suggested that the presence of wave-reflexion might be the cause of errors in deriving the pressure-gradient. In fact direct measurements of the phase shift of harmonic components of the pulse-wave at two successive points in arteries show that their apparent phase-velocities can be widely different. We consider that this is due to the presence of reflexions for reasons which are discussed in Section IV below.

This indicates that the pressure-gradient should be measured directly. To do this accurately requires a differential manometer, such as that used by FRY *et al.* (1956) in the proximal aorta. Even so measuring the gradient over a finite distance involves a consideration of the wave-velocity, i.e. the transmission time over this distance, but it can be shown that it is independent of the presence of reflexions.

The attraction of using the time-derivative was that matching of the two manometer calibrations could be avoided. The derivative may be obtained electrically but as a Fourier analysis has to be made anyway, it is as simple to differentiate each harmonic of the original record. The method is only satisfactory, however, if the apparent phase-velocities are also measured and it then loses any advantage of simplicity. A further defect is that it cannot measure the steady component of the pressure-gradient.

2.4. *The relation of the pulse-wave to the flow.* There has been some discussion in the physiological literature in the past concerning the phase relations of the pulse-wave and the flow. This is in spite of the fact that CHAUVEAU as early as 1869 (see HILL, 1900) showed that the flow peak preceded the pressure peak, and this has been confirmed many times (e.g. SHIPLEY *et al.*, 1943; MCDONALD, 1955). The phase difference varies with the size of the artery, being least in the aorta and increasing in the smaller arteries. The reason for this can be seen from the previous sections.

The flow lags behind the pressure-gradient by an angle of $(90 - \varepsilon)^\circ$ which is dependent on α (Fig. 3 and equation 12). The pressure-gradient is a function of the time-derivative of the pressure-wave and so will lead it by 90°. The flow curve will thus lead the pressure-curve by the value of ε approximately, and ε decreases with increasing α. At very small values of α on the other hand, this lead will approach 90°.

This is a minor point but is of some relevance when considering the pulsatile dilatation of arteries, for WOMERSLEY (1955a) has pointed out that this is determined by the phasic flow in an artery (equation 15), and not by the phasic pressure as is commonly assumed by analogy with static models.

2.5. *Other determinations of pressure-gradients and flow.* We have seen that the direct measurement of pressure-gradient excludes the errors of any assumptions made about the behaviour of the termination of an artery. A valuable technical contribution to this subject was made by SPENCER and DENISON (1956). They recorded the flow in various regions in the aorta of the dog, principally the thoracic descending aorta, with an electromagnetic flowmeter which appears to be the most reliable at present in the field (MCDONALD, 1958). At the same time they recorded the pressure-gradient by electrically differencing two strain-gauge manometers. These records have been

made available to WOMERSLEY who finds (personal communication) that the calculated flows are in fair agreement with the recorded flows. In view of the inherent inaccuracies of this method of recording gradients, this is encouraging. These results are also of interest because it is probable that a transient disturbance of laminar flow occurs in systole in an artery of this size. The Reynolds number calculated by the usual formula in which the critical number is 2000 (which is only strictly valid for steady flow) reaches a maximum of between 3500 and 4000 during systole. This suggests that short periods of deviation from laminar flow may be unimportant.

As mentioned above FRY has derived a simple equation from the Navier–Stokes equations and has used this to compute the flow in the root of the aorta from the pressure-gradient recorded with a differential manometer (FRY *et al.*, 1956). His formula is similar to an approximation for WOMERSLEY's equation when α is large and the terms M'_{10}/α^2 and ε are slowly approaching their asymptotic values. In practical terms this enables the flow to be determined by a simple analogue computer (FRY *et al.*, 1957) and avoids the calculations necessary by WOMERSLEY's method. Unfortunately they have no direct measurements of the flow in the aorta close to the heart with which the calculated curves may be compared. Their results are an interesting approach to the problem of measuring the form of the cardiac ejection flow pulse.

In summary, we can only repeat the opinion at the beginning of this section that there is no experimental evidence that can be said to have tested the theoretical analysis with any rigour. Work with models, however, gives reasonably satisfactory confirmation. In its application to the study of arterial flows in the living animal the theoretical predictions have also been confirmed to a reasonable degree of accuracy considering the instrumental problems in this field of physiology. Nevertheless confidence in the theoretical work rests largely on the knowledge that the physical analysis is realistic and any errors in predictions by the resulting equations must originate in the underlying assumptions, simple though they are. The validity of these assumptions (p. 123) must therefore be reviewed briefly.

2.6. *The validity of the hydrodynamic postulates applied to the arterial system.* 1. Laminar flow is known, on the evidence of dye studies, to occur in the smaller arteries up to the size of the dog femoral artery. Transient disturbances have been observed during systole in the abdominal aorta (MCDONALD, 1952b) and undoubtedly occur close to the heart due to the presence of the valves. The evidence on this subject is reviewed at length by MCDONALD (1958). It may be summarized by saying that turbulence is probably never established for sufficient length of time to cause a deviation from the pressure-flow relations (based on an assumption of laminar flow) that is large enough

to be measured with techniques used up to the present. The disturbances undoubtedly cause mixing of the blood but that is not especially relevant here.

2. The tube is long, i.e. end-effects are not considered. The inlet length for oscillatory flow is not known and this is a problem we look to physicists to solve. With the very flat profiles that would normally occur in the aorta (p. 128) this problem may not be of importance.

3. Anomalous viscosity. The vessels in which significant oscillatory flow occurs are all larger than those in which anomalous viscosity has been observed with steady flow. In tubes of arterial calibre there is evidence that blood behaves as a Newtonian fluid under oscillatory conditions (p. 130).

4. The absence of slip at the wall, i.e. the assumption that the lamina of fluid in contact with the wall has zero velocity, has never been disproved in any similar situation in fluid dynamics (GOLDSTEIN, 1938, Appendix). It may, therefore, reasonably be assumed to apply to the arterial tree.

5. The constancy of the radius of the tube under varying pressures constitutes a considerable assumption as it is common knowledge that arteries dilate with each pulse. However, the dilatation is small and the effect on the motion of the fluid has been calculated but appears to be too small to consider until we have further refinements in technique (p. 132).

There is, furthermore, the overall assumption in applying Fourier analysis to the arterial flow that the behaviour of the system is linear (Section I, p. 118). This is known to be only an approximation when considering the elastic tube. The interaction among the oscillatory components and between the oscillatory and steady terms has been investigated mathematically (WOMERSLEY, 1958a). As with the assumption 5 above, these non-linear terms are small in considering the flow of blood, but will need to be considered if a high degree of precision is ever attained in this field.

Throughout we can give only approximate answers to these questions but all the evidence available indicates that, complex as the arterial system is physically, the flow of blood can nevertheless be analysed relatively simply by the standard methods of hydrodynamics.

3. *The steady flow in arteries*

The fact that the arterial system is essentially a set of pipes for distributing blood should remind us that, functionally, the volume flow per unit time (the steady or mean flow) is the most important component. In the present analysis two aspects of the steady flow may conveniently be considered separately; (*a*) *the steady-flow component in the pulsatile flow* found in arteries, i.e. the mean term of the Fourier series, (*b*) *the*

flow out of the arterial bed which is all steady flow as the oscillatory components have been damped out.

3.1. *The steady-flow component in pulsatile flow.* If the shape of a typical flow curve (e.g. Fig. 6) is considered, it will be seen that it is an oscillation of asymmetrical shape. There is a large peak of forward flow, due to systole. In this case in the dog femoral artery the peak flow is 6·5 ml/sec (average velocity *c.* 80 cm/sec) while the mean flow is 0·85 ml/sec (velocity *c.* 12 cm/sec). Following this peak there is a reversal of flow leading to a backflow with a maximum value of 2 ml/sec. In terms of values about the mean we thus have a peak value of 5·65 ml/sec in the forward direction, and 2·85 ml/sec in the backward direction, i.e. towards the heart.

3.2. *Backflow.* Thus blood actually flows towards the heart during part of the cardiac cycle as a normal phenomenon in many arteries. Although SHIPLEY *et al.* (1943) recorded curves not dissimilar to that of Fig. 6 and reviewed much previous work that had shown backflow, the repetition of this observation by MCDONALD (1952a, 1955) has been disputed strongly. For example, RICHARDSON *et al.* (1952) stated that it could only be detected during marked vasoconstriction with adrenaline, while RICHARDS and WILLIAMS (1953) and SPENCER and DENISON (1956) stated that they could only record curves comparable with those of MCDONALD (1952a) by partially occluding the artery downstream. These disputes would have been more realistic if the essential components of the flow curve had been appreciated.

Pulsatile flow had been described here as oscillatory flow with a mean steady value. Backflow might thus be expected in all arteries and will only fail to appear when the steady flow is greater than the negative component of the compound oscillatory wave. Physiologically the appearance, or absence, of a backflow phase of flow will be determined largely by changes in mean flow which we can measure and predict. It will also depend on changes in the shape of the compound oscillatory flow curve of which at present we have much less certain knowledge. Thus the backflow phase in the abdominal aorta of the rabbit may be eliminated by increasing the mean flow through it. This was done either by creating a vasodilatation in the hind-part of the body or, equally well, by occluding large branches nearer the heart and thus decreasing the upstream "run-off" (MCDONALD, 1958, Chapter 7).

The significant difference between the "normal" femoral artery flow as recorded by RICHARDSON *et al.* (1952) and MCDONALD (1955) is not that the former did not observe backflow. Rather it is that with very similar mean flow rates RICHARDSON *et al.* (1952) only recorded a maximal oscillation between + 2·0 and + 0·1 ml/sec, whereas MCDONALD (1955), as we have seen, recorded an oscillation

between + 6·5 and — 2·0 ml/sec. That is the recording of the oscillation was very much smaller in the former case.

3.3. *The steady pressure-gradient.* The measurement of steady flow is thus an important factor in calculating arterial flow curves. In the arterial pressure gradient there is a steady term, which is related to the steady flow by POISEUILLE's formula. Calculating this flow would be simplicity itself were it not for technical difficulties in measuring the gradient. The mean pressure-drop along arteries is extremely small, e.g. the gradient corresponding to the mean flow in Fig. 6 (12 cm/sec) is only 0·13 mm Hg/cm. In the thoracic aorta, which is of about three times the radius and has, perhaps, twice the linear average steady flow velocity, the gradient is of the order of 0·03 mm Hg/cm. Thus for the whole length of the aorta the mean pressure fall is about 1 mm Hg at most. The measurement of such gradients in a system where the pressure is oscillating perhaps between 80 and 160 mm Hg during each cycle is far from easy.

In our own work, therefore, calculated steady flows have only been used in curves when they agreed with observed values. Where the pressure-gradient was derived from the time-derivative the steady component could not be measured and so an experimentally determined steady flow had to be added, as in Fig. 6.

3.4. *Theoretical considerations.* Non-linear interaction between steady and oscillatory terms must also be considered. This topic was dismissed briefly on p. 118 when it was stated that WOMERSLEY (1958a) had calculated the magnitude of this effect and found it to be small. This is so, but it should be added that it appears to be the largest of these small corrections. In one case for the femoral artery WOMERSLEY (1955a) found that the oscillatory terms might alter the steady flow by as much as 12 per cent. As the steady flow is itself small in comparison with the oscillatory flow an alteration of 12 per cent in it would change the shape of the flow-curve very little. In terms of mean pressure-flow relationships over the whole circulation it should be considered. WOMERSLEY's (1958a) later tabulations suggest that the overall effect is much less than that quoted. MORGAN and FERRANTE (1955) have also paid attention to this point and differ in detail from WOMERSLEY, but they were concerned with a steady flow which is large in relation to the oscillatory component—the reverse of the arterial condition.

3.5. *The total outflow from the arterial bed.* In the forms of the phasic flow-curve and the pressure curve in the femoral artery there is, ignoring the scale of the ordinates, an overall similarity. The most striking difference is in the mean values of both curves. The mean pressure is large in comparison with the size of the oscillatory terms while the mean flow is small. Thus the mean pressure in Fig. 1*b* is

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100 mm Hg and the maximal oscillation is from 80 to 140 mm Hg; by comparison, in Fig. 6 we see that the mean flow velocity is about 12 cm/sec and the oscillatory terms nearly 10 times that value (+ 85 to − 30 cm/sec). If we follow the mean pressure peripherally it falls very little until it reaches the smallest arteries and then over a very short distance it falls sharply to a value of about 30 mm Hg at the arterial end of the capillaries. The distance over which this fall in pressure of 50–60 mm Hg has occurred is short, though it cannot be precisely evaluated—almost certainly not greater than 0·5–1·0 cm. The mean pressure-gradient which was barely detectable in the arteries has become very large. In a capillary which averages 0·05 cm in length the pressure is considered to fall from 30 to 15 mm Hg approx.—a gradient of 300 mm Hg/cm. At the same time the flow has also slowed up tremendously so that while in an artery an average flow velocity of 10 cm/sec is common, that in a capillary is about 0·05 cm/sec. The calibres of all these vessels are very small, capillaries being of the order of 10 μ, so that the arterial tree may be thought of as terminating fairly abruptly in a diffuser. The data of SCHLEIER (1918) emphasizes the point that, although an artery branches fairly frequently the number of subdivisions increases enormously within the few millimetres before the capillaries are reached.

This account is familiar in any elementary text-book but it has implications that we would do well to consider. One functional reason for this arrangement is that fluid has to diffuse out of the capillaries to supply the tissue cells, and hence capillary pressure at the arterial end has to be above the osmotic pressure of the proteins of the plasma. Secondly, no vertebrate species has evolved a cardiovascular system that can simultaneously supply all its organs with a blood-supply sufficient for their maximal needs. The arteriolar system, controlled by smooth muscles, has been evolved, which enables blood to be diverted to regions of great need by reducing the supply to other regions. The point to establish here is that the arterial system in any region of the body ends quite suddenly in a large number of very fine vessels. For this reason the arterial tree is a high-pressure pipe line.

It is interesting to compare this with the venous system, the return half of the circulation, where we have a low-pressure line. The maximal pressures in this system are, in general, those at the distal end of the capillary, i.e. 12–15 mm Hg. The heart is in a chamber, the thorax, in which the pressure is a few mm Hg below atmospheric; the available gradient remains small, but veins are wider than arteries, and the total length of venules from a capillary bed is probably shorter than the corresponding arterioles. The two parts of the circulation at this level are thus very asymmetrical.

3.6. *Determination of the peripheral resistance.* When considering the

outflow through the capillary bed of a single region or of the whole arterial tree it is not possible to measure all the dimensions of the system that are required for applying POISEUILLE's formula. If we consider the physical relations that this equation includes we can write the pressure and flow relations as

$$(P_1 - P_2) = K \,.\, Q$$

where $K = 8\,\mu L/\pi R^4$ from equation 1. By analogy with Ohm's law we can call the constant K the fluid resistance of the system. For a given vascular bed it is usually assumed that changes in resistance are due to changes in the radius of the arterioles. Surrounded as they are by smooth muscle whose activity is controlled by nervous and hormonal mechanisms, there is little doubt that these vessels are mainly responsible for active physiological changes in resistance. However, while there is a pressure-drop from about 80–85 mm Hg in the smallest arteries to 5–10 mm Hg in small veins, across the peripheral vascular bed, it does not by any means all occur in the arterioles. The pressure in the proximal end of the capillaries is at least 30 mm Hg. Therefore of a total pressure difference of 70–80 mm Hg some 20–25 mm Hg fall occurs in small vessels, the capillaries and venules, beyond the arterioles. Under some experimental conditions designed to test the variation in size of the vessels of the peripheral resistance, e.g. by increasing venous pressure as in the work of PHILLIPS *et al.* (1955), changes found may not necessarily involve the arterioles.

Blood is known to show anomalous viscous properties in small vessels. WHITTAKER and WINTON (1933) found that the viscosity of blood perfused through the vascular bed of a dog's hind limb was only 2·5 times that of water, whereas in large tubes it is about 6 times this value. PAPPENHEIMER, for example, has laid particular stress on this factor as a cause of change in resistance with changing rates of flow (PAPPENHEIMER and MAES, 1942; PAPPENHEIMER and SOTO–RIVERA, 1948).

It is not proposed to review the extensive literature on physiological changes in the peripheral resistance. The examination of the stability of the components of this resistance has been carried out in a series of papers by LEVY and his colleagues. LEVY *et al.* (1954) studied the mean pressure-flow relationship in the whole systematic circulation of the dog. The total cardiac output was measured by a standard technique using the Fick principle with measurements of the oxygen saturation of mixed venous and arterial blood. The mean arterial pressure was recorded and regarded as a measure of the pressure difference across the peripheral vascular bed; the venous pressure, which is always small, was treated as being at atmospheric pressure. Over a mean arterial pressure range of from about 40 mm Hg to 160 mm Hg they

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found that, when the pressure-receptor reflexes from the carotid sinus and aortic arch regions were eliminated, there was a linear relation between pressure and flow. However, in animals with these reflexes intact there was a marked fall in peripheral resistance with increasing pressure, i.e. reflexly mediated vasodilatation was occurring.

This indicates that over the physiological range of mean arterial pressures the vessels, in which the main peripheral resistance occurs, remain stable and may be treated as a linear system. In the intact animal, however, compensating mechanisms decrease the resistance as the pressure rises.

In a single limb of an animal the factors playing a part in the peripheral resistance were studied in more detail. PHILLIPS *et al.* (1955) found that for a constant arterio–venous pressure difference the flow was greater as the venous pressure, i.e. the transmural pressure in the small vessels, was increased. This implied that they distended with increased transmural pressure. LEVY (1956) further showed that this effect was found whether blood or a homogeneous liquid (dextran) was used so that the increased flow was not due to anomalous viscosity effects of the blood. These changes in resistance were not very great and as they were not found with changes of arterial pressure alone in the experiments on the total systematic bed, it is possible that the vessels that were distended were on the venous side of the arterioles.

Many studies have been made of the relation of the mean calibre of the small "resistance vessels" to the mean pressure within them. W. M. BAYLISS (1902) originally suggested that increasing the tension on the smooth muscle in the arteriolar walls caused it to contract. That is, a rise in pressure would cause a vasoconstriction. FOLKOW (1949) and FOLKOW and LÖFVING (1956) have more recently supported this view with extensive experimental results. *A priori* it seems illogical to emphasize the physiological dominance of this mechanism, since it would appear to act as a positive feedback loop and so be completely unstable. Thus a rise in blood pressure would cause a vasoconstriction which would cause a further rise in pressure.

The relevance of these experiments to the physical laws governing flow lies in the assumption (5 on p. 124) that the radius of vessels is independent of the pressure. We have already noted that the pulsatile change in calibre in arteries has a very small effect on the flow within them. Changes dependent on the mean pressure in small vessels could produce markedly non-linear pressure-flow relations. WEZLER and SINN (1953) have made a special study of this and have derived a modified version of Poiseuille's formula which incorporates a term whereby R is dependent on P. Their experiments appear to have been all done on the pulmonary vascular bed where the effect is possibly greater than in the systemic vascular bed. Until more agreement is

reached on this topic it would seem simpler to accept the results of LEVY *et al.* (1954) which suggest that variations in vascular dimensions due to changes in pressure are not due to direct effects in the vessels but due to active physiological mechanisms, namely, the moderator reflexes. In this case they may be experimentally excluded when studying the physical characteristics of the vascular bed.

The behaviour of the arterioles when the pressure within them is lowered has been the subject of an interesting analysis by BURTON (1951). He showed that a vessel with a wall that was composed of a mixture of elastic material and actively contractile muscle would be expected to become unstable at a certain critical pressure. The vessel would then close completely and this point of instability BURTON has called the "critical closing pressure." While some work has apparently confirmed the existence of the phenomenon, whereby flow through an arteriolar bed will cease at a pressure appreciably above the venous pressure (see BURTON, 1954) other workers have failed to confirm this, e.g. LEVY *et al.* (1954). While the physical analysis demonstrates important aspects of wall-tension problems in the arterial tree its direct application, or at least experimental verification, in the arterioles depends on the biological properties of the smooth muscle and so falls outside the scope of the present review.

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4. *The behaviour of the arterial wall*

Reference has already been made to some of the conclusions reached by WOMERSLEY (1955a, 1958a) on the effects of elastic behaviour of the wall of a tube on the motion of the liquid within it. This analysis has recently been published in full detail. The investigation has emphasized the interrelation of many aspects of physiological phenomena in the arterial system that we need to consider.

The first model of an artery considered was that of a thin-walled elastic tube, containing a viscous fluid in oscillatory motion. It was thus similar to the model treated briefly by KARREMAN (1952) and more fully by MORGAN and KIELY (1954). These workers were concerned only with the problem of wave-transmission, and found that the wave-velocity was frequency-dependent, and that travelling oscillations were attenuated; that is, the wave-velocity was a complex quantity. WOMERSLEY has computed curves exhibiting these effects, for tubes of sizes and frequencies in the physiologically important range (Fig. 9*a*, *b*). Discussion of the application of these results is deferred to Section III.

The movements of the wall are also of physiological interest. It is well known that arteries dilate with each cardiac ejection, although this change in size, when measured, is smaller than appears to the eye. Thus, even the thoracic aorta does not seem to vary more than $\pm$ 3–4

per cent of its mean radius under physiological conditions; the abdominal aorta has been recorded as varying by $\pm$ 1·5 per cent (LAWTON and GREENE, 1956) and the femoral artery by not more than $\pm$ 2 per cent (see MCDONALD, 1958). Most experiments on arterial elasticity have been done by distending isolated segments with static pressures, but WOMERSLEY (1955a) has pointed out that in the body the arterial expansion is more directly related to the flow. This relationship may be written

$$\frac{2\xi}{R} = \frac{\bar{w}}{c} \qquad \ldots.(15)$$

where $\bar{w}$ is the average velocity of flow, c the wave-velocity (complex), ξ the radial displacement, and R the mean radius of the artery. As the peak pulsatile flow always leads the peak arterial pressure in phase, this results in a tendency for the maximum dilatation to occur in advance of the maximum pressure, as has been reported by RUSHMER (1955). In addition to the radial movement, the viscous drag of the fluid will also cause a longitudinal movement of the wall of a free elastic tube. This effect should in fact be larger in magnitude than the radial movement. Careful study of arteries *in situ* has, however, failed to show the predicted longitudinal movement. Further observations have shown that the arteries are largely tethered by the connective tissue around them, and it appeared clear that it was not entirely realistic to use the free elastic tube as a model.

The next model considered was therefore that of an elastic tube subjected to an external longitudinal restraint, and loaded by the mass of the tissues around it (WOMERSLEY, 1957). This set of conditions was summarized mathematically in terms of a constant K. In the case of the free elastic tube this represented the ratio of wall-thickness (h) to the radius (R), so that in WOMERSLEY (1955a) we have

$$K = h/R \qquad \ldots.(16)$$

In arteries at a mean arterial pressure of about 80 mm Hg, $K \sim 0{\cdot}15$. It has been found to be remarkably constant, from the aorta down to the smallest artery studied, the saphenous of the dog (MCDONALD, 1958). Curves have been computed up to $K = 0{\cdot}4$ and the differences are of no practical importance. The Poisson's ratio (σ) of the arterial wall is taken to be 0·5, as LAWTON (1954) found that arterial strips stretched isovolumetrically.

WOMERSLEY (1955a) has also calculated the behaviour of the wave-velocity in tubes where $\sigma = 0{\cdot}25$ and 0·00.

With the "tethered and loaded tube" (WOMERSLEY, 1957) the term K is defined thus,

$$K = \left(1 + \frac{h_1\rho_1 R_1}{h\rho R}\right)\left(1 - \frac{m^2}{\omega^2}\right) \qquad \ldots.(17)$$

where h = wall thickness and h_1 = the thickness of the loading mass, with ρ, ρ_1 and R, R_1 the corresponding values for the density and the radius. m = natural frequency of the longitudinal restraint and ω = circular frequency of the oscillation. With a fairly stiff constraint, as we appear to have in the body, $m \gg \omega$, and K is negative, tending to $-\infty$ as m is increased indefinitely.

The effect of this modification of wall-behaviour on wave transmission is shown in Fig. 9. It can be seen that the asymptotic value of the phase-velocity is now greater than that for a non-viscous fluid in the same tube, and that damping has increased. Both these effects are related to what is, in essence, an increase in stiffness of the tube. Longitudinal movement due to viscous drag cannot occur, while the only effect on the radial movement is to modify its phase-relationship to the pressure. This result has been applied by WOMERSLEY (1957) to the data of LAWTON and GREENE (1956), with fair agreement. The "tethered and loaded tube" is thus the most satisfactory model of the artery that has so far been analysed in detail. With a stiff restraint ($K < -10$) the equations for the motion of the liquid become indistinguishable from those of the rigid tube first considered, but the wave-velocity remains finite.

The treatments of the problem which have been reviewed above have assumed that the wall of the tube was composed of a Hookean elastic substance. It is known, however, that this is quite a severe approximation to make regarding the arterial wall, although for small pressure variations about any given mean value its behaviour may reasonably be considered to be linear. Other theoretical treatments have been proposed to take into account some of the peculiar properties of the arterial wall but, being mainly concerned with wave-velocity, are more suitably discussed in Section III.

5. *Arterial impedance*

Because the analysis of alternating current flow in electrical circuits is more familiar to most physicists than the problems of oscillatory fluid flow, it is not surprising that analogies have been drawn between the two (LANDES, 1949; RONNIGER, 1954; TAYLOR, 1957a). Like all analogies this one is useful provided it is not pressed too far, so that similarities are treated as identities. The use of the term "resistance" in discussing steady flow is unexceptionable, and by analogy we may refer to "fluid impedance" for oscillatory flow. For example, from equation 11 for flow in a rigid-walled tube, under a pressure-gradient $-Ae^{i\omega t}$, we have the average velocity

$$\bar{w} = \frac{Q}{\pi R^2} = \frac{AR^2}{i\mu} \cdot \frac{M'_{10}}{\alpha^2} \cdot e^{ie_{10}} \cdot e^{i\omega t} = Ae^{i\omega t} \cdot \frac{M'_{10}}{i\omega\rho} e^{ie_{10}} \quad \ldots.(18)$$

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If we make use of the analogy of average velocity and electrical current, with pressure and voltage taken as equivalent, then the longitudinal impedance of the tube per unit length is

$$Z = \frac{A e^{i\omega t}}{\bar{w}} = \frac{\omega\rho}{M'_{10}} \sin \varepsilon_{10} + \frac{i\omega\rho}{M'_{10}} \cos \varepsilon_{10} \qquad \ldots . (19)$$

Although these expressions do not, as one might expect, explicitly contain the coefficient of viscosity or the radius, these are implicit in the quantities M'_{10} and ε_{10}, which are both functions of α, which is itself a function of μ and R. It can be seen that for high frequencies, the impedance approaches $i\omega\rho$, that is, the motion of the fluid is governed by its density only, and the impedance is a pure inductance. WOMERSLEY (1958a) has determined these quantities also, in the form of a factor which multiplies the Poiseuille resistance term $8\mu/R^2$; the form adopted above is otherwise identical.

The input impedance (Z_0) of a conducting system depends both on the longitudinal impedance (Z) and the wave-velocity (c), and is defined as

$$Z_0 = Z \,.\, c/i\omega \qquad \ldots . (20)$$

For the tethered and loaded tube (WOMERSLEY, 1957) this leads to

$$Z_0 = \frac{\rho \,.\, c_0}{\sqrt{(1 - \sigma^2)}} \cdot \frac{1}{\sqrt{(M'_{10})}} \cdot \exp\left(- i\tfrac{1}{2}\varepsilon_{10}\right) \qquad \ldots . (21)$$

It can be seen that with increasing α, as M'_{10} tends to 1 from below, and ε_{10} tends to 0 from above, the input impedance approaches that for the inviscid system except for the factor $\sqrt{(1 - \sigma^2)}$, which is not taken into account in the development of the "water-hammer" formula (equation 3). In a perfectly elastic system c_0 denotes the value given by the Moens–Korteweg formula; if this term is constant, then the modulus of the input impedance decreases with increasing frequency of oscillation to an asymptotic value. However, if the wall material is elasto-viscous, then "c_0" will not be constant, but will rise with frequency, so counteracting the tendency for the input impedance modulus to decrease, or even leading to its increase.

5.1. *The input impedance of a vascular bed.* We have already discussed the peripheral resistance of a vascular bed, as determined by the total pressure-drop across it and the steady flow through it. In the same way, we can characterize its properties in respect to pulsatile flow, by determining its input impedance. The values obtained for this will not however be given by the relations set out above, which apply only for a system without reflexions, but will depend on the magnitude of any reflected components which are present, and the distance of the

site of reflexion from the point of observation. Fig. 7 shows the variation of the modulus of the input impedance of a segment of rubber pipe with variation in its length, while the frequency is kept constant. When the occlusion was at a distance of a quarter-wavelength the input impedance was at a minimum, while at a half-wavelength it was at a maximum. For a system of fixed length, with variation in frequency, minima and maxima of the input impedance modulus will occur when the length represents respectively an odd or even multiple of a quarter-wavelength. The converse holds for a tube with an open end.

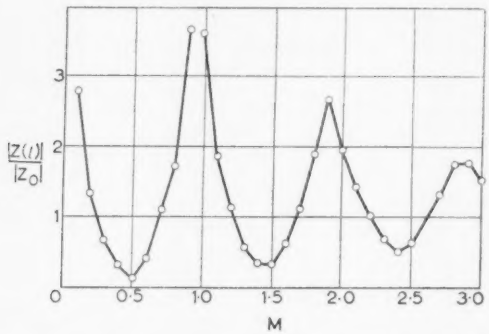

Fig. 7. The ratio of input impedance moduli, $|Z(l)/Z_0|$; $Z(l)$ is for a rubber tube occluded at a distance l from the origin, and Z_0 for the "infinite" tube. The frequency was 7 c/s, and the wavelength approximately 2m. It can be seen that the input impedance modulus has maxima and minima at quarter-wavelength intervals, and in this resembles the behaviour of apparent phase-velocity (cf. Fig. 11) (from TAYLOR, 1957b).

The significance of these changes in impedance is simply related to the fact that when a sinusoidal oscillation is reflected, the resultant wave shows points of maximum and minimum amplitude (anti-nodes and nodes) at quarter-wavelength intervals, and that an anti-node of pressure will always coincide with a node of flow. It is easy to see that at a closed or partially closed end the pressure oscillations will be at a maximum, and the flow oscillations at a minimum, so that the impedance itself is at a maximum. A quarter of a wavelength away there will be a "node" of pressure and an "anti-node" of flow, so that the impedance will be at a minimum. These conditions will alternate as one moves away from the reflecting-point, but owing to the influence of damping the variations will become progressively smaller. The ratio of the maximum of the impedance to the true value will depend on the magnitude of the reflexions. When the influence of reflexions is considered in more detail in Section IV, it will be seen that the measured phase-velocity of an oscillation also varies in this manner.

In the arterial system the only requirements for calculating this

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quantity are simultaneous recordings of pressure and flow. Fig. 8 shows such a recording, together with the impedances for the first 8 harmonic components plotted as a function of frequency. From this it can be

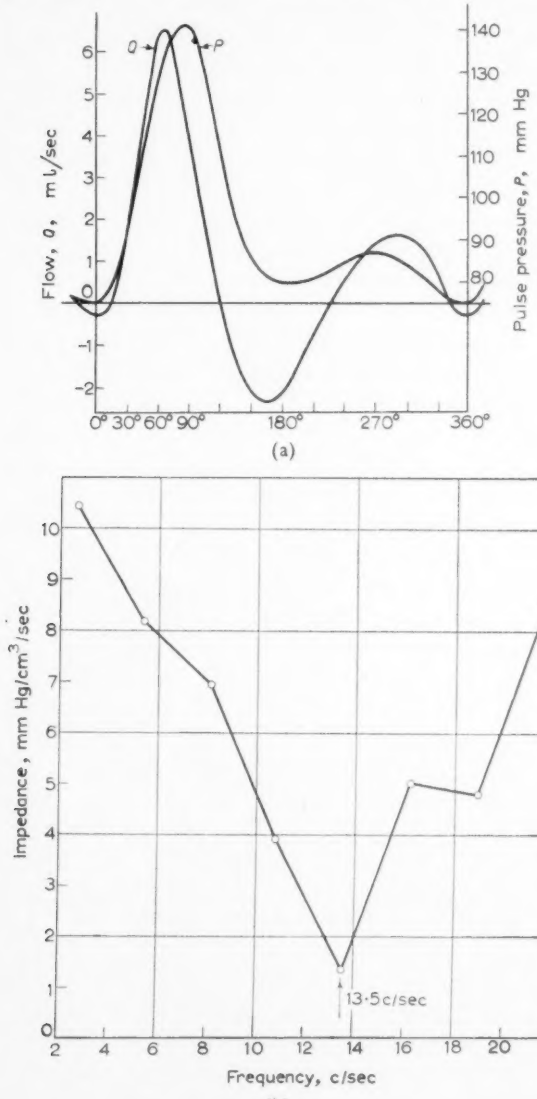

Fig. 8. (*a*) Pulsatile flow and pressure recorded simultaneously in a femoral artery of the dog (from McDONALD, 1955). (*b*) The modulus of the impedance presented by the femoral vascular bed, calculated from the recordings in (*a*) for the first 8 harmonic components and plotted as a function of frequency. The fundamental frequency is 2·7 c/s.

seen that the impedance was at a minimum for the frequency 13·5 c/s. For still higher frequencies, the modulus of the input impedance rose again. This shows that the fall in the lower frequency range was not due to a decrease in input impedance of the kind indicated by equation 21; in the first place, over this range of frequencies theory does not predict a fall of more than 6 per cent, and secondly it cannot account for the subsequent increase. It seems clear, therefore, that the minimum can best be explained as being due to the presence of a reflecting-site which at 13·5 c/s is a quarter-wavelength distant from the measuring point. As the wave-velocity in the femoral artery is between 8 and 10 m/sec (Dow and Hamilton, 1939) this places the average reflecting-point some 15–20 cm away, i.e. between the knee and the ankle. As the peripheral resistance is usually expressed in terms of pressure and volume flow, we have employed the same units in this example. In the theoretical treatment above, and in some other situations, the input impedance may be derived in terms of average velocity of flow.

Randall and Stacey (1956b) have measured pressure and flow in the femoral artery using an electromagnetic flowmeter applied to a cannula, and have calculated the impedance of the femoral vascular bed. They state that it is at a minimum at about 3 c/s, but the description of their apparatus (Randall and Stacey, 1956a) indicates that their pressure-recordings were made from a point about midway along the cannula, and so perforce they measured the impedance of the cannula plus the leg; this probably explains the discrepancy between their results and that given above. In any case the impedance minimum they found implies a reflexion site 60–80 cm distant, which is impossibly far.

In studies on arterial pulsatile blood-flow, a great deal of attention has been paid to the development of instruments that will not only record flow accurately, but which will disturb as little as possible the normal pattern of flow. If impedance measurements are made, however, provided that the flow is accurately measured, some deviation from the normal pattern is permissible, since the pressure which is simultaneously measured is that which results from the actual flow into the region under study. The ratio of pressure to flow, giving the input impedance, should, within reasonable limits, be independent of the forms of pressure- and flow-pulses. It is important, however, that the pressure be measured at the origin of the vascular bed, i.e. at the distal end of the cannula. When arterial flows can be directly recorded, calculation of the input impedance is, as we have seen, a straightforward matter, only involving a Fourier analysis of the pressure and flow-curves. Alternatively, use may be made of the methods set out in Section II, 1, whereby flow may be calculated from the pressure-gradient (Bergel *et al.*, 1958) and so used to derive the impedance. The method is yet in its infancy, but

the use of the measurement of arterial impedance should provide the most simple way of describing the properties of the peripheral vascular bed in relation to pulsatile flow.

III. Wave-travel in the Arterial System

It has long been recognized that the wave-velocity in a fluid-filled pipe such as an artery is dependent on the elastic properties of the wall (equation 2). More recently attention has also been drawn to its dependence on the frequency of the oscillation, due to the viscosity of the liquid (Fig. 9), and also to the viscosity of the wall-material. In the foregoing sections, it has been shown that the wave-velocity is a fundamental parameter of the arterial system; it is involved in the relations between the form of the pulse-wave and the pressure-gradient (equation 14) and hence in the oscillatory motion of the blood. Furthermore it is involved in the equation (15) relating the pulsatile expansion of an artery to the flow within it.

1. *The factors determining the rate of wave-travel*

In a tube with a thin, perfectly elastic wall (Young's Modulus E), and filled with a non-viscous fluid of density ρ, the rate of travel of a disturbance is given by the Moens–Korteweg formula (equation 2)

$$c_0 = \sqrt{(Eh/2R\rho)}$$

where $h/2R$ is the ratio of wall-thickness to diameter. According to this formula, waves of all frequencies are propagated at the same velocity and are not attenuated in travel. In fact, of course, the influence of fluid viscosity must be taken into account in real systems. This has been done in the treatments of Karreman (1952), Womersley (1955a) and Morgan and Kiely (1954), for an unconstrained elastic tube where the "wave-equation" for c has been obtained by determining the consistency of the set of equations for wall- and fluid-motion, with the necessary boundary conditions on each. The solution of the wave-equation, c, is a complex number, and is a function of the parameters $\alpha = R\sqrt{\frac{\omega}{\nu}}$, $K = h/R$, and the Poisson's ratio of the wall-material, σ. In Womersley's treatment, $c_0/c = (X - iY)$, where c_0 is the value given by the Moens–Korteweg formula. The real and imaginary quantities in this expression determine the phase-velocity and the attenuation of the travelling wave.

The phase-velocity of the disturbance, c_1, is given by $c_1/c_0 = 1/X$, and the attenuation per wave-length by $\exp(-2\pi Y/X)$. Both these quantities are given in Fig. 9 from computations by Womersley. From these results one sees that as α increases (i.e. for any given tube, as $\sqrt{\omega}$ increases) the phase-velocity c_1 in a free elastic tube, approaches

the value c_0. This finding is related to the motion of the fluid within the pipe; at high values of α this is determined by the inertial properties, since the effects of viscosity become relatively less important,

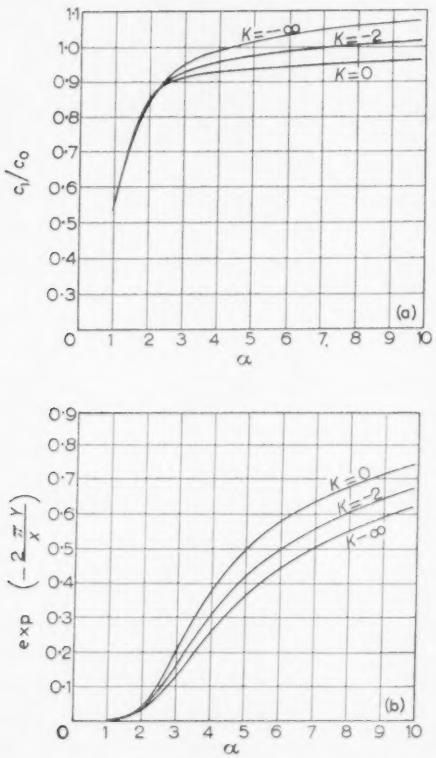

Fig. 9. (*a*) Variation of phase-velocity, c_1, with α in a perfectly elastic tube, subject to varying degrees of constraint and filled with a viscous liquid. c_1 is expressed as a fraction of c_0, the wave-velocity given by the Moens–Korteweg equation (2). K is defined in equation 19; $K = 0$ represents a very thin unconstrained tube, $K = -\infty$ a tube with complete longitudinal restraint, and $K = -2$ one with a small degree of constraint. (*b*) Variation in damping with α, under the same conditions as (*a*). The expression $\exp(-2\pi Y/X)$ gives the fraction to which the amplitude of a wave is reduced after travelling one wavelength. As the wave-length decreases with frequency, the damping *per unit length* increases (from WOMERSLEY, 1957).

so that the situation approaches that for which the Moens–Korteweg formula is valid, i.e. for a non-viscous fluid.

The experiments of MÜLLER (1951), confirmed by those of TAYLOR (unpublished), indicate that in rubber pipes the phase-velocity continues to increase with frequency, and does not approach a limit as these treatments suggest. HARDUNG (1952b) in discussing MÜLLER's results,

has attributed this to the influence of internal viscosity in the wall. This explanation is supported by the observation that a change in the value of α due to a change in the viscosity of the liquid within the pipe did not alter the phase-velocity nearly as much as the same change in α due to a change in frequency. The arterial wall is known to be elasto-viscous, and the recent observations of LANDOWNE (1957a) that high-frequency oscillations (up to 50 c/s) are transmitted along a length of umbilical artery at velocities which continuously increase with frequency and greatly exceed the normal values for the pulse-wave velocity, are almost certainly explicable on this basis. LANDOWNE (personal communication) has made similar observations on the wave-velocity in the arteries of the human arm. HARDUNG (1953) has made a detailed study of the dynamic elastic constants of aortic specimens which lead to the same conclusions (see Section I, 3).

The travel of oscillations in tubes of rather more special kinds has been discussed by several other authors. LAMBOSSY and MÜLLER (1954), dealt with a thick-walled tube composed of an anisotropic substance, and obtained expressions for modifications of the term E' to be inserted in the Moens–Korteweg formula which take account of the wall properties and dimensions. MORGAN and KIELY (1954) have studied wave propagation through a tube loaded by external mass, and WOMERSLEY (1957) has discussed the behaviour of the "tethered and loaded tube," where in addition to mass-loading, the wall of the tube is under longitudinal elastic restraint. This model appears to be the most realistic one yet proposed for an artery *in situ*, but additional modification may be required to take into account the internal viscosity of the wall.

MORGAN and FERRANTE (1955) and WOMERSLEY (1958a) have studied in detail the effect of a steady stream on the propagation of oscillations. For practical purposes provided $\bar{V} \ll c_0$ it appears that the phase-velocity is increased simply by addition of the stream-velocity, so that $\tilde{c} = c_1 \pm \bar{V}$ depending on whether the disturbance is travelling with or against the stream. The experiments of MÜLLER (1950) do not entirely support this prediction, but the discrepancy is probably due to the manner in which MÜLLER determined the velocity. It appears from his paper that he timed the passage of the advancing front of a wave-train which, owing to the effect of dispersion is not the appropriate measure.

KING and LAWTON (1950) have explored the elastic properties of many biological tissues, including arteries, in terms of elastomeric theory. They have used the model of a tube with an elastomeric wall since the arterial wall was found to resemble rubber, in that its elastic behaviour depended on the degree of extension from its "resting-length." The term "elastomer" in this context appears to be restricted

to this property; viscous effects, such as were separately considered by LAWTON (1955) are not included in the definition of an elastomer, although elastomeric substances also show them. KING (1947) obtained an expression for the variation of pulse-wave velocity with variation of mean arterial blood pressure. This dependence is due to the rise in effective elastic modulus with increasing distension of the arteries by an increasing internal pressure. The rate of increase of pulse-wave velocity with mean pressure was found to be characterized by an "age-dependent" parameter β related to the postulated polymer chain-length. This approach seems very interesting in following the behaviour of the arterial system over wide ranges of pressure but does not include the wall- or fluid-viscosity which, by making the wave-velocity frequency-dependent, also plays an important role.

2. *What is the true arterial wave-velocity?*

Although the measurement of the pulse-wave velocity would seem to be one of the simplest procedures in circulatory physiology, it is necessary to define what it is we wish to measure. On the basis of the Moens–Korteweg equation (2) the main emphasis in the past has been on relating the pulse-wave velocity to the elastic behaviour of the arterial wall. As the wave-velocity, as measured by the rate of travel of the foot of the wave (the foot-to-foot velocity) increases as the wave travels centrifugally, this has been taken as evidence that the more peripheral arteries have a much higher modulus of elasticity than those near the heart. If, on the other hand, the *phase-velocities* of the harmonic components of the pulse-wave are recorded, it is found that they may be greatly increased by the presence of reflexions. If the effects of changing wall properties and of reflexion are to be distinguished, we must know what the wave-velocity would be in an artery in the absence of reflexions. Furthermore, owing to the elasto-viscous properties of the wall, and the viscosity of the blood, the "true" wave-velocity will depend on the frequency of the oscillation. This latter consideration is of particular importance if, having located a reflecting-point at, say, a quarter-wavelength distance from the point of observation, we wish to find the actual distance.

2.1. *The velocity of the "foot" and other points.* It is clearly desirable to have a method for measuring the pulse-wave velocity which is both simple and informative; the rate of travel of a point on the front of the wave, such as its foot, has been the standard method for many years, and is probably still the best single method. Although the dispersion of the wave due to viscosity (see Fig. 2*a*) introduces certain inaccuracies, the pulse-wave velocity is usually measured over relatively short distances, and this factor may reasonably be neglected. The wave is, however, being markedly distorted by reflections as it travels, and

hence the identity of any particular feature must not be taken as self-evident.

The foot of the wave is a somewhat indefinite point from which to measure short transmission times accurately, so that other manoeuvres are sometimes made, to establish a sharply-defined, yet recognizable "point of correspondence." KAPAL *et al.* (1951) have reviewed the validity of the method introduced by FRANK in which the velocity of a point one-fifth of the height of the rising limb of the wave is measured. These authors found that the velocities of the foot, and of four "corresponding points" on the rising limb near to it, were not appreciably different. LASZT and MÜLLER (1952a) extrapolated the rising limb as a straight line, and measured the point of intersection of this line with the pressure at the foot.

It has, however, long been recognized that if other points higher up on the curve are used, their measured velocity may be very different from that of the foot. WIGGERS (1928) pointed out that whereas in one example the foot of the wave in the aorta had a velocity of 3·2 m/sec the shoulder preceding the incisura of the wave travelled at 17·6 m/sec. This, and similar observations, have been attributed to the increase of the elastic modulus of the artery as its internal pressure increases. This non-linear behaviour was first studied in detail by ROY (1880), and its application to pulse-wave velocity was developed by BRAMWELL and HILL (1922), who showed the dependence of the foot-to-foot velocity on the end-diastolic arterial pressure. The extension of this pressure-velocity relationship to individual points on the curve led to the prediction that the point of maximum pressure would be travelling at a much higher velocity than the foot of the wave. BRAMWELL and HILL (1923) carried this idea even further, when they suggested that after travelling a certain distance the peak must overtake the foot, and said that "under suitable conditions, parts of it may actually tend to topple over, and form breakers." The analogy between waves in an artery filled with blood and waves on the ocean does not appear very safe, and has not proved fruitful. In fact, the apparent rate of travel of the point of maximum pressure may be less than that of the foot. For example, HALE *et al.* (1955) found peak-to-peak transmission velocities throughout the dog aorta and in the femoral artery that were only about half the values for foot-to-foot velocities that were recorded in the same regions by DOW and HAMILTON (1939) and by LASZT and MÜLLER (1952a, b). Further unpublished work (by TAYLOR) on simultaneously recorded aortic and femoral waves, obtained over a wide range of pressures and heart-rates caused by vagal stimulation, has shown that in general the peak-to-peak velocity was only 75 per cent of the foot-to-foot velocity; furthermore while the peak-to-peak velocity in both regions was strongly correlated ($p < 0{\cdot}001$) with the diastolic

pressure it showed no significant relationship to the pulse-pressure and hence to the systolic pressure. The foot-to-foot velocity showed similar relationships.

The inherent fallacy of regarding the pressure-level of individual points on the wave as determining their velocity may perhaps best be shown by measuring the apparent velocity at points of equal pressure on the rising and falling parts of the curve. In any wave which alters its shape as much as those shown in Figs. 1 and 10 it is obvious that such points will have widely different "transmission times."

In terms of steady-state oscillations such as we have been using, it is to be seen that the foot of the wave, like any of its other features, is determined by its harmonic composition. In general one may say that any sharp inflexions in a wave-form are determined by the higher frequency components. The phase-velocities of these components measured over the usual intervals are relatively stable; this is probably the reason why the foot-to-foot velocity gives fairly consistent results. Under conditions where reflexions are large, we must consider the possibility that the foot-to-foot velocity will be affected by them.

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Measurements of this velocity in various regions of the arterial tree show a marked increase in the peripheral arteries. In the case shown in Fig. 10 this velocity increased from 400 to 600 cm/sec as the wave passed from the thoracic to the abdominal aorta. In the femoral artery this is much higher, and is about 1000 cm/sec. The range of results in LASZT and MÜLLER's (1952a, b) experiments is approximately 400–500 cm/sec in the thoracic aorta, 570–850 cm/sec in the abdominal aorta and 850–1300 cm/sec in the femoral artery. These figures are in good agreement with those obtained by DOW and HAMILTON (1939). These changes in velocity are usually ascribed to an increase in the elastic modulus of the arteries concerned. It should be appreciated however, that from the Moens–Korteweg equation this requires an increase in modulus of from 1·5 to 2·0 times between thoracic and abdominal aorta, and a further increase by 2–4 times from abdominal aorta to femoral artery, because the ratio of wall-thickness to diameter remains virtually constant in all these vessels (HÜRTHLE, 1920). There are few satisfactory measurements of the elastic moduli of arteries and it has become necessary to reconsider this point in the light of the estimations of the composition of the arterial wall by HARKNESS *et al.* (1957) who found that for all arteries except the smallest, elastin and collagen together constituted about 50 per cent of the dry weight. The relative proportions of elastin and collagen, however, were markedly different between the thoracic and abdominal regions of the aorta. In the former, there were about twice as much elastin as collagen in the wall; in the abdominal aorta, and indeed in all extra-thoracic arteries, these proportions were reversed. As collagen is much less extensible

than elastin, it is easy to understand how this change in composition might account for a rise in elastic modulus in the abdominal as compared with the thoracic aorta. The further large increase that appears to be present in the femoral artery would, in the absence of any change in composition, have to be explained purely by a change in the arrangement of these fibres and there does not seem to be any clear histological evidence for this. Therefore we must consider the possibility that the increase in foot-to-foot velocity in the femoral artery may be due to the influence of reflexions. A hint that this might be so can be drawn from the observation of BAZETT and DREYER (1922) that the wave-velocity is much more variable in peripheral than central arteries and is "in all probability much more dependent on local conditions of vasoconstriction or dilatation." Such evidence as we ourselves have obtained is equivocal, and so for the present we accept the assumption that the foot-to-foot velocity is a fair measure of the true phase-velocity of at least the higher-frequency components of the pulse-wave. Lacking any precise knowledge of what the true velocity of the low-frequency components may be, we have used the measured foot-to-foot velocity for purposes of illustration and for making estimates of wave-lengths.

2.2. *The velocity of a superimposed impulse.* LANDOWNE (1957b) has developed a very neat method of studying the elastic behaviour of the arterial wall by the use of a short-transient pressure-pulse, obtained by tapping the artery with a solenoid-operated metal plunger. He then measured the transmission time of the spike (10 msec duration) between the brachial and radial arteries in man. He thus eliminated any possible effects of reflexion, because with such a short spike he was only recording the centrifugal passage of the impulse. Like other workers, LANDOWNE found that the impulse travelled faster as the arterial pressure increased. This was also true for individual spikes generated during the high-pressure period of the pulse. The velocities he recorded were, however, all much higher than those usually found in human arteries. They ranged from 10 m/sec at a transmural pressure of 30 mm Hg up to about 30 m/sec at 180 mm Hg whereas BRAMWELL *et al.* (1923) found a value of 2·6 m/sec at 30 mm Hg and BRAMWELL and HILL (1922) reported a normal range of 4–10 m/sec with a predicted maximum of 18·5 m/sec at 200 mm Hg. Similarly BAZETT and DREYER (1922) found a velocity of 4 m/sec in the brachial and 8·5 m/sec in the radial artery. A study of the illustrative curves given by LANDOWNE, also suggests that the spike was "overtaking" the pulse-wave, because it was travelling faster.

The explanation that we advance for this phenomenon is based on the known elasto-viscous behaviour of the arterial wall. A sharp spike of this type contains principally high-frequency components of the order 100–200 c/s. The elasto-viscous wall is much "stiffer" for frequencies

of this order than for the low range of frequencies which we have so far been considering as the harmonic components of the pulse-wave. A stiffer artery will naturally have a much higher wave-velocity, hence a spike will travel faster than a low-frequency wave. This relationship between frequency and wave-velocity has itself been most clearly illustrated also by LANDOWNE (1954, 1957a and private communication), in experiments where he used a similar apparatus to that described above, but which imposed a sinusoidal oscillation on the exterior of the artery. From 5 or 6 c/s up to 50, or even in some cases 100 c/s, he found a steady rise in wave-velocity. In the brachial artery of man he found that there was a minimum at about 5 c/s, and that the wave-velocity tended to rise again as the frequency was reduced to 2 c/s. As the sinusoidal oscillations were maintained long enough for the effects of reflexion to occur, this rise at low frequencies is almost certainly the same phenomenon of increased apparent phase-velocity seen in the normal pulse-wave (Section IV). With an oscillation of small amplitude the higher frequencies probably have their reflexions damped out and the phase-velocity would then be determined entirely by the elastic behaviour of the wall.

If this is so, and the general agreement between the velocity of high-frequency sine waves and isolated spikes supports the contention, then this velocity is one of the values of true phase-velocity that we have been seeking. It will be, in fact, the value for the frequency at which it is determined, because we can see from Fig. 7*a* that the effect of the viscosity of the fluid on wave-velocity is small at $\alpha > 3$. For the human brachial artery at 50 c/s, $\alpha \sim 20$. These methods of LANDOWNE's, therefore, are an important step forward in measuring the elastically-determined wave-velocity in arteries.

They leave unsolved, however, the problem of determining the true phase-velocity for the frequency-range of 1–15 c/s, in which as we shall see we are especially interested for estimating the changes in apparent phase-velocity due to reflexions. The only way to determine this "true phase-velocity" appears to be by measuring the elastic constants accurately and deriving the wave-velocity from them. This involves using excised arterial specimens, which is naturally less desirable than studies made in the body, but considering the very large effects of measured wave-velocity caused by reflexions it seems unavoidable that this should be done.

IV. REFLEXIONS

The marked change in the form of the arterial pulse-wave, which occurs as it travels peripherally from the heart, has intrigued cardiovascular physiologists for a long time. In particular it increases in amplitude, exhibiting the so-called "peaking," and develops secondary

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waves; the causes of these appearances have given rise to considerable speculation, and the explanations provided by the Windkessel theory have already been discussed in Section I, 1. In steady-state analysis, however, the form of the travelling pulse-wave must be considered in terms of its harmonic components. It has been found, for example, that the amplitude, especially of the lower-frequency terms, increases peripherally, as is illustrated in Fig. 10. In addition we find that the phase relationships vary so that the apparent phase-velocity may be greatly elevated over its expected value. There seems little reason to doubt that these findings are due to the presence of wave-reflexion, and the analysis of these effects is the main concern of this section.

1. *The sites of reflexion*

In a conducting system where reflexion is occurring, both advancing and retrograde waves will be present, the pressure at any point being their resultant. Both waves, if one neglects any influence of a steady streaming of the liquid, will be propagated with the same velocity, and with the same rate of attenuation. By analogy with cable theory, we may employ the term the propagation constant, which is a function of the properties of the system and the frequency of the oscillation. It is related to the wave-velocity by the equation

$$\lambda = i\omega/c \qquad \ldots.(22)$$

If at some point the properties of the system change, then a reflexion will be set up there, and a retrograde wave will travel thence. Any change will bring this about; for example, HARKNESS *et al.* (1957) have directed attention to the change in elastic composition of the aortic wall at the level of the diaphragm; this point of transition to a stiffer-walled tube could be a source of reflected waves. Reflexions will also occur at points of branching; ALEXANDER (1953) for example, has stressed the importance of the diaphragmatic region as a source of reflexions, by reason of the number of branches at that level to supply the viscera.

The reflexion coefficient R at a junction is defined as

$$R = \frac{Z_T - Z_0}{Z_T + Z_0} \qquad \ldots.(23)$$

where Z_0 is the characteristic impedance of the parent line, and Z_T is the impedance presented by the branches. This demonstrates that unless $Z_T = Z_0$ then a reflexion will occur, so that the reflected wave P_2 is a certain fraction of the incident wave P_1. Since in general these quantities are complex the two waves will differ not only in amplitude but also in phase.

In Section II, 5, the longitudinal impedance of a rigid tube was

given by equation (19). WOMERSLEY (1957) has shown that in the limiting condition of longitudinal constraint, the same expression applies to the elastic tube; taking account of the wave-velocity in such a tube, WOMERSLEY (1958b) has used equation (21) to derive an expression for the ratio Z_0/Z_T, where a parent-vessel of radius $\underline{R}$ gives rise to two identical branches, of radius $\underline{r}$. Denoting the Moens–Korteweg values of the wave-velocity on either side of the junction by c_1 and c_2, he has calculated curves for three conditions (*a*) $c_1 = c_2$, (*b*) $(c_2/c_1) = \sqrt{(\underline{R}/\underline{r})}$ and (*c*) $(c_2/c_1) = \underline{R}/\underline{r}$. We see that $2(\underline{R}/\underline{r})^2$ is the ratio of the cross-sectional area of the two branches to that of the parent-vessel, and WOMERSLEY has plotted the reflexion coefficient in terms of this area-ratio for each of the three cases. His results show that no system of this kind can be entirely free of reflexions, since it is not possible to match exactly the impedances of the two systems on either side of the junction, although this may be achieved to within one or two per cent. For bifurcation of arteries there is probably an increase in wave-velocity as in case (*b*) or (*c*), so that we find the area-ratio for best matching should lie between 1·18 and 1·30. BLUM (1919) found the area-ratio of arterial division in general to lie between 1·2 and 1·4, while HESS (1927) found it to be 1·126 for the bifurcation of the aorta in man. From experimental results in the dog WOMERSLEY (1958b) calculated that the reflexion at the bifurcation of the aorta would amount to 14 per cent with a phase angle of 40°. Applying similar calculations to the multiple branching in the upper abdominal aorta WOMERSLEY predicted a reflexion of 7 per cent, with a phase-angle of almost 90°. This is tending towards a more "open-ended" type of reflexion, but is far less than the complete inversion that was postulated by ALEXANDER (1953) for this region.

In the examples just considered it was assumed that the branches continued indefinitely, or that their terminations were such that no reflexions arose there. In the arterial system these conditions are not fulfilled, and the behaviour of reflexions at junctions must depend not only on the characteristics of the branches arising there, but on the nature and location of successive branches and their final terminations, which are generally agreed to be of a "closed" type.

The magnitude of the "peripheral reflexion" cannot be estimated directly, but from measurements of apparent phase-velocity it can be determined approximately (TAYLOR, 1957a). For the fundamental frequency of 2·5 c/s, the reflexion at the terminations in the femoral vascular bed is approximately 50 per cent under normal conditions, and may be larger. RONNIGER (1954) also estimated it to be of this order. An interesting problem which remains to be studied is the degree to which reflexion from a given set of arterial terminations is determined by the anatomical architecture of the small branches, and how

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much it varies with the varying constriction of the arterioles under changing physiological conditions. At present there are no data of sufficient accuracy to warrant an estimate of these factors, but it seems from the calculations of WOMERSLEY given above, that the total reflexion in a vascular bed is much greater than that from the sites of branching of major arteries.

In the arterial system the distance between successive branching-points becomes less and less as the order of branching increases, and although one cannot precisely locate the ultimate reflecting site, anatomical dimensions dictate it within certain limits. In the dog, for example, the furthest arteriolar bed of the hind-limb is within 40–50 cm of the aortic bifurcation, so that the average distance from this point to the main sites of reflexion within the hind-limb will be considerably less.

2. *The moduli of the harmonic components*

The combination of a reflected with an incident wave, as we have seen, determines both the phase and the amplitude of the resultant oscillation. As was discussed in Section I, the amplitude will have maxima and minima at points along the system, spaced approximately a quarter-wavelength apart. Two things are needed for the production of absolute nodes of pressure in a conducting system; these are, complete reflexion, and freedom from attenuation. Neither of these conditions is found in the arterial system, so that the retrograde wave will necessarily be of smaller modulus than the advancing wave and the cancellation required for the production of absolute pressure-nodes is impossible. Maxima and minima may occur as the result of partial cancellation but the ratio between them, because of the attenuation, will change as one moves away from the reflecting point. Thus it is not possible to apply the standard electrical concept of a standing-wave ratio, since the proportion of the wave which is standing depends on the point of observation. These considerations apply whether the end of the system is "open" or "closed," but it is possible to differentiate these two cases by studying the change in modulus of the pressure within a quarter-wavelength of this point. The pressure will increase over this interval towards a closed termination which establishes a relative node of flow and hence an anti-node of pressure. This has been demonstrated in the rubber-tube model by TAYLOR (1957b). Conversely it will decrease towards an open end.

In view of statements that a "node" of pressure for the whole pulse-wave exists in the abdominal aorta, it should be remarked that the above description applies only to single harmonic oscillations. For a compound wave such as the pulse, it would be necessary on this basis to postulate a set of standing harmonic waves with coincident

nodes. This coincidence could only occur at intervals of a whole wavelength of the fundamental in an ideal system. Owing to the presence of viscosity we have seen that the wave-velocity is frequency-dependent so that the wave-lengths of the harmonic components are not simple fractions of the fundamental. Further, at the "periphery," all oscillations are probably at a relative anti-node, and since the whole arterial system represents about a quarter-wavelength for the fundamental oscillation, the coincidence of nodes within this distance is not possible. Although pulsations in the arterial system have been discussed in the physiological literature in terms of "standing waves" we feel that this expression is neither helpful nor exact. Variations in amplitude of this type can be illustrated by an analysis of the beautiful recordings of LASZT and MÜLLER (1952a, b) in which the pressure was measured simultaneously at four different points in the arterial system. Fig. 10 reproduces 4 curves recorded at intervals of 12 cm along the aorta of a dog. A Fourier analysis of all curves in their original time-relations was made with the foot of the curve measured in the ascending aorta taken as the origin. The shapes of the individual curves are displayed and above them in Fig. 10 are plotted the moduli of the first 6 harmonic components of each wave.

The first point to note is that the first and second harmonics are far larger than any of the others. As the energy of a sine-wave is determined by the square of its modulus we can see that as $M_1 = 8{\cdot}0$ and $M_2 = 5{\cdot}2$ in the ascending aorta then $(M_1^2 + M_2^2) = 91$, and as the sum of the squares of the moduli for all 6 harmonics is only 103·9 these two harmonics account for almost 90 per cent of the energy, even in the central aortic pulse. More peripherally the percentage is higher. Secondly we see that all the moduli tend to rise as the wave passes peripherally.

Considering individual components it can be seen that the modulus of the fundamental rises steadily over the whole length of the aorta. This is to be expected as throughout it is within a quarter-wavelength of the major reflecting sites. The second harmonic remains more or less constant along the thoracic aorta but then rises very definitely, and the addition of an occlusion in the femoral artery causes a further sharp rise in this harmonic. We would anticipate a minimum of pressure for this component at the quarter-wavelength position which might be expected to fall in the thoracic aorta and one may indeed have fallen between the proximal recording points. The peripheral rise of the second harmonic is always marked in the femoral pulse and is the main contributory factor to the appearance of the dicrotic wave (Fig. 1*b*). The higher harmonics, however, show no waxing and waning to indicate the positions of relative nodes.

The pattern does not illustrate with any neatness the clear-cut

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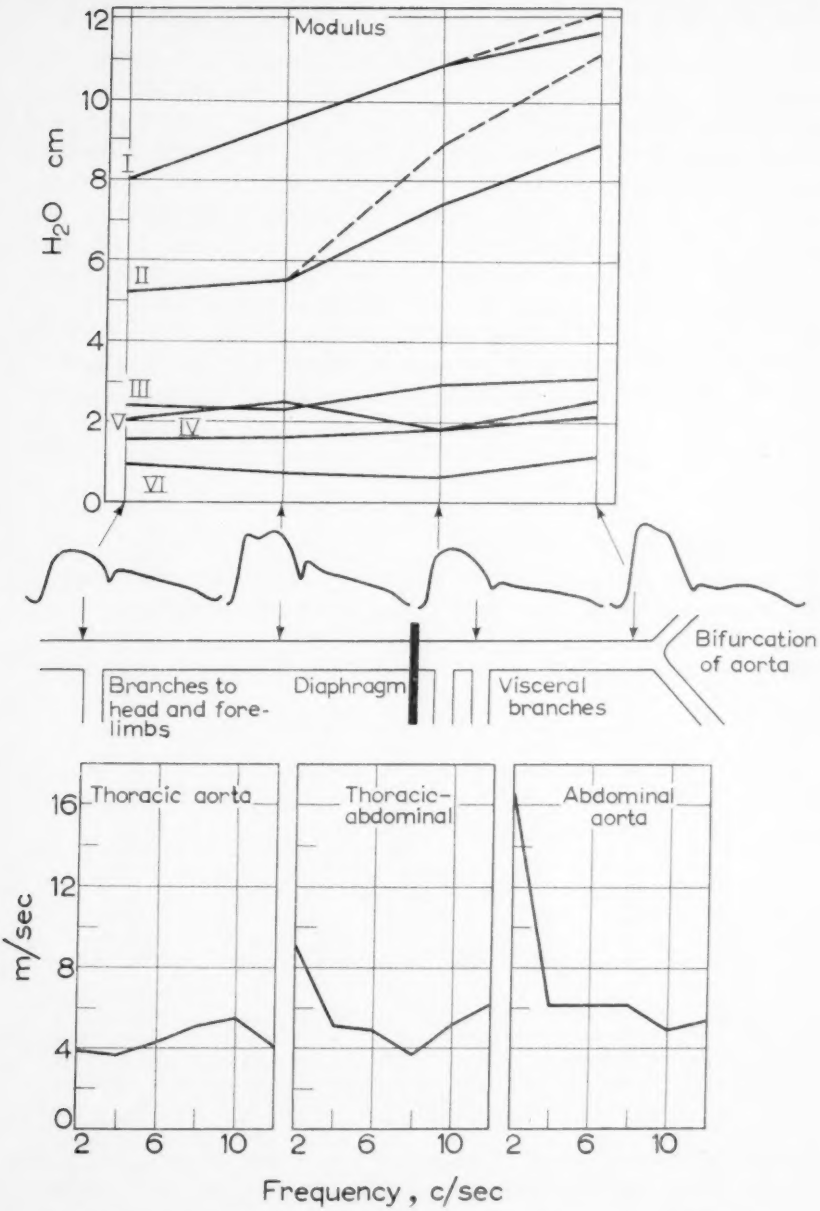

Fig. 10. The analysis of four pressure recordings made simultaneously in the aorta of a dog. The individual pulse-forms are shown, but have not been corrected for small differences in manometer calibrations. The moduli of the first 6 harmonics are plotted above (corrected). The dotted lines show the effect of occlusion of one femoral artery. Below the formalized diagram of the aorta are the apparent phase-velocities, measured over the three intervals. These results are discussed in the text. (The pulse recordings are from Fig. 11 of LASZT and MÜLLER, (1952a), and used by kind permission of the authors.)

distribution of maxima and minima that one would wish, but in view of the fact that reflexion does not occur at any one point but rather a series of such points distributed in depth, it at least shows no marked discrepancies from that predicted by theory. One definite conclusion may be drawn from the results in Fig. 10. It is that the increase in the moduli as the wave passes peripherally points inescapably to the conclusion that the termination is of a "closed-end" type. There is no suggestion at all of an open-ended type of reflexion in the region between the bifurcation of the aorta and the femoral artery such as postulated in the Windkessel models of WEZLER and BÖGER (1939) and SINN (1956) which were discussed in Section I, 2. Neither is there any suggestion of "the standing wave in the aorta" that was described by HAMILTON and DOW (1939) and by ALEXANDER (1953) or of the open-end reflexion in the upper abdominal aorta that the latter author postulated.

3. *The apparent phase-velocity*

In considering reflexions it is informative to discuss what is meant by the phase-velocity of a wave containing a reflected component. First, the phase-velocity of a simple non-reflected wave is given by measuring the space rate-of-change of its phase, i.e. $\mathrm{d}\phi/\mathrm{d}x$, the phase-gradient,

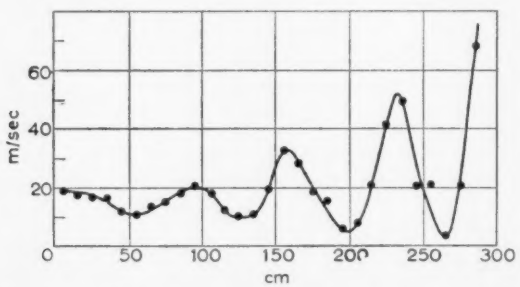

Fig. 11. Apparent phase-velocity (m/sec) determined over 10 cm intervals along a pipe occluded 300 cm from the origin. The frequency was 10 c/s. The phase-velocity as measured oscillated about its true value in the unoccluded pipe of 14·1 m/sec. One wave-length is approximately 140 cm (from TAYLOR, 1957b).

so that for a simple wave, writing $\lambda = u + iv$, and denoting the phase of $P(x)$ by ϕ, then if $P(x) = P_0 \exp(-\lambda x + i\omega t)$ we have $\mathrm{d}\phi/\mathrm{d}x = -v$, so that $c = -\omega/(\mathrm{d}\phi/\mathrm{d}x) = \omega/v$. However, for a wave with a reflected component, this term cannot be so conveniently expressed, since $P(x) = (P_1 \mathrm{e}^{-\lambda x} + P_2 \mathrm{e}^{+\lambda x})$ and the equation for c no longer takes a simple form. Like the impedance, c now depends upon the distance of the point of observation from the reflecting site, and on R. Thus, it is better to refer to this velocity as the *apparent phase velocity*. Fig. 11

shows the results obtained in a length of rubber tube occluded at one end. As in any experimental situation the phase shift must be determined over a finite interval, the apparent phase-velocity is denoted by $\bar{c}'$ to distinguish it from the theoretical c' which is its value at a point. As can be seen the apparent phase-velocity is greatly increased near the occlusion. Observations made at increasing distances from this point showed alternate maxima and minima of $\bar{c}'$, in the same manner as a theoretical example derived by TAYLOR (1957a). It was shown there that if measurements are made within a distance of rather less than a quarter-wavelength from the reflecting point, then the apparent phase-velocity c' is always in excess of the true value c, found when $R = 0$, whether the ending be open or closed. It can also be seen in Fig. 11 that the further away from the reflecting point the less the discrepancy of $\bar{c}'/c$; this results from the change in the proportions of incident and reflected waves present, due to the influence of damping.

By recording the pulse-wave simultaneously at two points a short distance apart, one may find the phase-differences between the harmonic components by Fourier analysis, and these phase-gradients may be used to determine the phase-velocities of the oscillations. The first measurements of this kind appear to be those of PORJÉ (1946) and were made in human beings. He recorded the pulse-wave in the subclavian and femoral arteries and the abdominal aorta, by external application of a piezo-electric crystal transducer. His results were only analysed for 3 harmonics, and in all cases he found that the phase-velocity of the fundamental oscillation, and often also of the second harmonic, was markedly higher than the usual values found by measurement of foot-to-foot transmission time. He correctly attributed this phenomenon to the influence of reflexions. KAPAL *et al.* (1951) have repeated his experiments, and compared the results with foot-to-foot velocities. They also found very high phase-velocities for the fundamental, but general agreement between the foot-to-foot and the phase-velocities of the third harmonic. The significance of the behaviour of the higher harmonic values in experiments of this type is difficult to assess, since the measurements were made over such large intervals.

Measurements of apparent phase-velocity over much smaller intervals (7–10 cm) have been made by MCDONALD and TAYLOR (1957); the results of such an experiment are shown in Fig. 12. It can be seen that the fundamental at 3 c/s has a phase-velocity considerably in excess of that found by foot-to-foot measurements. As was found theoretically, and in the rubber-pipe model (Fig. 11) apparent phase-velocities are elevated by the presence of reflexions, if measured near the reflecting site. The expectation for wave-velocity in a dispersing system of this type is that it should always increase, however slightly, with increasing frequency (cf. Fig. 9), whereas in our experiments the reverse was the

case. Similar results are displayed in Fig. 10 where the apparent phase-velocities are plotted below each interval as a function of frequency. In the thoracic aorta the value at 2 c/s is 390 cm/sec and tends to rise with frequency, up to 10 c/s, where the value is 555 cm/sec (the fall again at 12 c/s may be due to uncertainty in the analysis of a component of small amplitude). This is the type of phase-velocity distribution that might be expected in the absence of reflexion. Between the lower thoracic and upper abdominal aorta, however, there is a marked change in the variation with frequency. The apparent velocity at 2 c/s has increased to over 900 cm/sec and there is a minimum at 8 c/s and then a further increase. This suggests that there is a major reflecting site at a distance of a quarter-wavelength for 8 c/s. This distance is obviously less than a quarter-wavelength for the lower frequencies and greater for the higher: both situations which cause an increase in apparent phase-velocity (Fig. 11). Finally, in the abdominal aorta we see that for the lowest frequency $\bar{c}'$ is now nearly 1700 cm/sec and the minimum is in the region of 10–12 c/s. The "foot-to-foot velocity" is here 600 cm/sec, and if we may take this to be the "true" wave-velocity of this region of the arterial system, it means that the wave-length of this frequency is 50–60 cm and so the quarter-wavelength position of the minimum would place a major reflecting site some 12–15 cm away, although it does not correlate well with the minimum in the second curve. This suggests that the terminations of the large vessels supplying the pelvic viscera and perhaps the thigh muscles are the dominant reflecting site in relation to the aorta.

If one or more major vessels are occluded there can be no doubt that we are creating a closed end point of reflexion. Such occlusion should, therefore, cause a further increase of the apparent phase-velocities that we find under normal conditions in the arterial tree. Results of an occlusion of this type are shown in Fig. 12 for the abdominal aorta. It will be seen that the phase-velocities are markedly increased by clamping the iliac arteries. A similar effect was found in some other curves of LASZT and MÜLLER (1952a) where a marked increase in phase-velocity, especially at 4 c/s, occurred in the abdominal aorta due to occlusion of one femoral artery.

The fact that the velocity of the higher frequencies is changed very little by a major occlusion is puzzling, but the relative stability of the phase-velocity of oscillatory terms of 10 c/s and above has frequently been found in our experiments. It was found (Fig. 10) under normal conditions and in the same experiment when one femoral artery was clamped. This is partly to be explained by the fact that the phase-velocity represents the average over a finite interval. In Fig. 10 this was 12 cm and in Fig. 12 it was 8 cm. At the highest frequencies in the two examples (again taking 600 cm/sec as the wave-velocity) in the

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abdominal aorta the interval in both is about one quarter-wavelength. From Fig. 11 it can be seen that this distance will always involve a region where $c'/c_0 > 1$ and where $c'/c < 1$ so that there is an averaging effect that brings $\bar{c}'$ closer to the velocity in a tube unaffected by reflexion. However, close to a reflecting-point this averaging effect would not be expected entirely to cancel out changes in apparent wave-velocity.

The conclusion seems to be that if one wishes to analyse the reflexion effects on wave-velocity one must take as short an interval as is

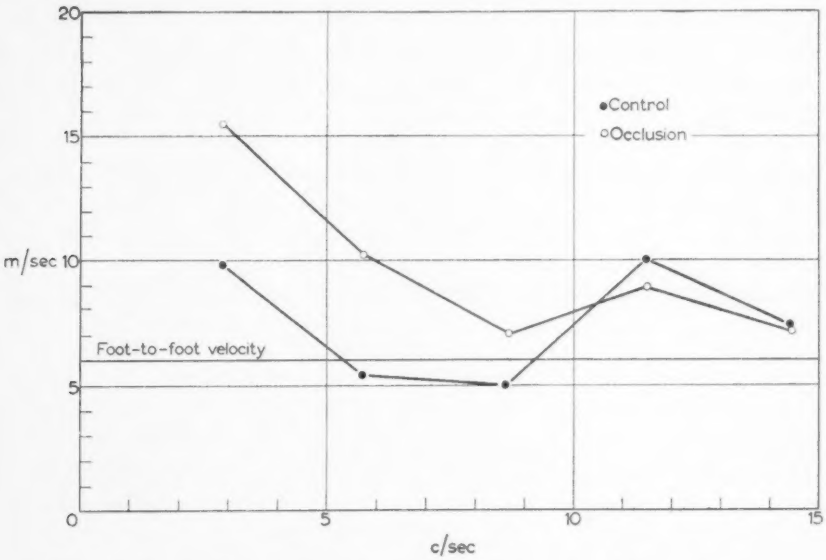

Fig. 12. The apparent phase-velocities of components of the pulse-wave recorded in the abdominal aorta of the dog before and during occlusion of both common iliac arteries. The high apparent phase-velocity of the fundamental component is attributed to the influence of reflexions; this is supported by its further increase when additional reflexion is created.

technically possible. If on the other hand the main point of investigation is the elastic behaviour of the wall, then taking a longer interval will reduce the discrepancies between the measured phase-velocities and their "true" values. The length of the interval is then only limited by the size of the region it is wished to study.

We must also consider the effect on phase-velocity that may result from the fact that the elastic modulus of the arterial wall increases with increasing distension. We have noted that the "foot-to-foot" velocity is correlated with the diastolic pressure; in the case of phase-velocity we must regard the mean pressure as determining the effective working

level. The modulus of each harmonic determines its maximum pressure variation from this mean and in Fig. 10 it can be seen that the largest oscillation is only $\pm$ 12 cm H_2O, i.e. only about 10 per cent of the mean pressure. The mean is usually closely related to the diastolic pressure, and one might therefore expect that the true phase-velocity would vary with the arterial pressure in much the same way as does the "foot-to-foot" velocity. The changes in wall-behaviour due to fluctuations of pressure within one cycle will depend on the resulting distensions. In considering simple harmonic oscillations, we cannot speak of different velocities of the peak and trough of the wave as a cause of distortion, but must express it rather in terms of the generation of higher harmonics. In a compound wave this would involve interaction between the harmonic components. There is, as yet, no information on the magnitude of this effect. Comparative studies of the static elastic behaviour of thoracic and abdominal aorta and the femoral artery suggest that this non-linearity of behaviour increases as we move away from the heart, and this would increase harmonic interaction peripherally, as was put forward by KAPAL *et al.* (1951), who considered that a disturbance originally purely sinusoidal in the thoracic aorta would appear distorted at the periphery.

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V. SUMMARY AND CONCLUSIONS

Our aim has been to present the point of view that the arterial circulation can be regarded as a system in a steady state of oscillation. The advantages of this concept are several; in the first place it unifies the consideration of pulsatile pressure and flow changes, since methods are described for deriving the one from the other; in the second place it emphasises the need for simultaneous measurement of more than one variable, if the properties of the arterial system are to be determined; thirdly, the number of simplifying assumptions is very small, namely, stability of the heart-frequency over a sufficient interval of time, and that for small displacements about the mean the equations of the system are linear; and finally, that predictions based on theory and on model experiments are reasonably well satisfied.

In the course of this presentation we have found it necessary to examine and to criticise the Windkessel concept, which we consider to be founded on some severely limiting assumptions. The treatment of the pulse as a transient phenomenon which this model requires is, of course, theoretically possible, but the analysis that such a treatment involves is far more complicated than the advocates of this approach appear prepared to employ.

The number of previous workers who have employed steady-state analysis in these problems is small, and the potentialities of the method

have by no means been fully exploited. As we have tried to show, however, there is now sufficient justification, both theoretical and practical, for proceeding to their thorough experimental development and application.

The relation of oscillatory pressure-gradient and flow has been derived and found to apply satisfactorily in arteries. All other measurements have been shown to be modified by the presence of reflected waves. This necessitates the making of more than one measurement at a time. If a recording of the oscillatory pressure is obtained simultaneously with a direct measurement of the flow it is possible to calculate the input impedance (for each harmonic component) of the region into which the flow is occurring; the variation of input impedance with frequency being known, one may obtain information on the reflexion conditions at the arterial terminations. Or, if pressure and pressure-gradient be measured together, it is possible to calculate the flow from the gradient and thus find the impedance indirectly. From two pressure measurements made simultaneously a short distance apart, or by using the time- and space-derivatives of the pulse-wave one may find the apparent phase-velocities of the components, and hence obtain some information on reflexion conditions. These effects depend on the magnitude of the reflexion, and its distance from the point of observation. When this distance is considered in terms of the wave-length of the oscillation concerned, it is of interest to note that the arterial system as a whole is about a quarter-wavelength of the fundamental component of the pulse.

The problem of the true value of wave-velocity in the arterial system has been discussed, since the influence of fluid and wall viscosity will render it frequency-dependent. The presence of reflexions does not permit its determination directly from the pulse-wave, except, probably, for the higher frequency components. It is suggested that the conventional "foot-to-foot" velocity may be a useful measure of their true velocity. In this case, however, the expectation is that the true velocity of the lower-frequency components must be somewhat less than the measured "foot-to-foot" velocity. In this connexion the dynamic elastic behaviour of the arterial wall is of particular interest as in order to find the true wave-velocity of these low-frequency components, it may be necessary to derive it indirectly from measurements made on arterial specimens.

In conclusion, while there are several aspects of these matters which have not yet received sufficient attention, for example the possible influence of non-linear wall behaviour, the general concept of the arterial system in a steady state of oscillation seems to be sufficiently established to permit and encourage experimental investigation on these lines.

ACKNOWLEDGEMENTS

J. R. WOMERSLEY, whose untimely death occurred during the preparation of this review, was closely associated with the initiation and development of the studies in this Department; we wish to record our appreciation of his invaluable collaboration, and our sorrow at the loss of a friend and colleague.

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4

DETERMINATION OF MOLECULAR DIMENSIONS FROM LIGHT SCATTERING DATA

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4

DETERMINATION OF MOLECULAR DIMENSIONS FROM LIGHT SCATTERING DATA

Anton Peterlin

I. Introduction

In recent years light scattering by dilute solutions (Debye, 1944)* has proved to be one of the most used methods for the determination of the size and shape of macromolecules and colloid particles, i.e. of their mol. wt. and of one characteristic length (gyration radius). The theory of the effect is very simple and can be easily understood. The main advantage of the method is the rather simple experimental equipment required, which is either commercially available at low cost or can be built from inexpensive parts. In sharp contrast with electron microscopy where the particles are completely dried and dead, one has the possibility of studying the particles in a medium which does not appreciably differ from the normal environment in the living cell and to investigate the influence of temperature, different solvents, *p*H and ionic strength. On the other hand there are also some severe limitations. Due to the intense interaction effects the measurements have to be made at extremely low concentrations, for only then can an unambiguous extrapolation to zero concentration be performed, yielding information on the single particle. As a rule these concentrations are more than 10 times lower than the similar ones in intrinsic viscosity measurements. Further, the particle dimensions can only be determined when they are not too small as compared with the wave-length of light. Diameters less than 300 Å cannot be determined. In some cases the gap between 300 Å and the range accessible by the usual X-ray techniques can be covered by small angle X-ray scattering.† A very important condition for the experiment is the extreme purity of the sample, for any dust particles with their enormous scattering power completely deform and screen the effects of the solute.

* The method was first applied by Gans (1919) to colloidal suspensions, later by Putzeys and Brosteaux (1935) to protein solutions. Its general use as a standard method for determination of mol. wt. and particle size, however, started with Debye (1944). For further information see the review articles by Zimm *et al.* (1945), Oster (1948), Doty and Edsall (1951), Edsall and Dandliker (1951), Stuart (1952, 1953, 1955), Peterlin (1953a), Oster and Pollister (1955), Stacey (1956).

† See for instance Fournet and Guinier (1950).

II. The Basic Concepts of the Scattering of Light

A perfectly crystalline arrangement of atoms or molecules does not coherently scatter light having a wave-length which is larger than twice the longest identity period of the basic cell. For this reason the ideal crystal scatters X-rays but not visible light whose wave-length is nearly 1000 times too large. Hence the scattering of visible light by a real crystal is entirely due to the imperfections of the crystal, i.e. to *optical density fluctuations*. As liquids are much less ordered than solids they exhibit larger density fluctuations and accordingly scatter light much more than solids. Dissolved molecules or suspended particles represent permanent deviations from the mean optical density of the solvent and hence act as additional scatterers of light.

The *amount* of light scattered by a single dissolved molecule or suspended particle is proportional to the square of the *induced excess electrical moment* in the particle.* The excess moment is proportional to the excess polarizability of the particle and to its volume, the proportionality factor still depending on the shape and orientation of the particle and on the polarizability of the particle and the solvent. In a given solvent–solute system and random orientation the scattered intensity per particle turns out to be proportional to the square of the volume V of the particle. A volume of 1 ml of solution containing N independent particles yields an excess scattering intensity proportional to $N \,.\, V^2$. The concentration c in grammes of the solute per ml equals $N \,.\, V \,.\, \rho$. Hence the ratio of the excess scattering intensity to the concentration, i.e. the specific scattering, is proportional to the volume, V or the mass, $m = V \,.\, \rho$, i.e. the molecular weight, M of the single particle.

As the dimensions of the particle approach $\lambda_0/2\pi n$ the phase difference between different parts of the particle cannot be neglected in the calculation of the scattered intensity. The simple summation of the amplitude remains strictly valid for zero scattering angle only. Hence for obtaining the volume or mass of the particle one has to *extrapolate* the scattered intensity to the *angle zero*. At any other angle the phase differences reduce the scattering (Fig. 1). The angular dependence of the scattered light hence provides a means for evaluating the particle dimensions. For this determination one has to assume a special model (sphere, rod, random coil) which is mostly known already from other investigations. Without any assumption of this kind only the subsequent moments of the intraparticular distances, i.e.

$$\langle r^2 \rangle = 2\rho^2 (\rho = \textit{gyration radius}), \langle r^4 \rangle \ldots$$

* Excess moment means the moment due to the excess of polarizability of the particle over that of the solvent.

can be obtained. Thus the measurement of the *intensity* and *angle dependence* of the excess scattering due to the solute yields the average size and shape of the dissolved molecules or suspended particles.

Instead of measuring the whole angular dependence one can only determine the ratio of the scattered light at two angles, $90 - \alpha$ and $90 + \alpha$ (*angular dissymmetry*) or the ratio at two different wave-lengths but at fixed angle, mainly at 90° (*dispersion* of the scattering) and

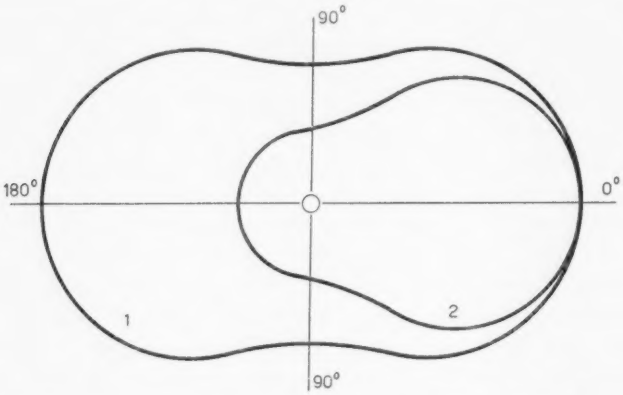

Fig. 1. Angular distribution of the light scattered by a small (1) and a large (2) particle having the same excess moment.

deduce from it the dimensions of the particle according to the model chosen.

The intensity of light scattered by the particle in solution is proportional to the product of $1/\lambda^4$ and a function of the ratio x/λ where x is a characteristic length of the particle. With variation of λ both factors change rather markedly. In order to avoid superposition of so rapidly varying scattering functions one usually measures with monochromatic light.* As an exception of this practice suspensions of rather large spherical particles may be mentioned where the colour changes (higher order Tyndall spectra) of the scattered light are used for determination of the sphere diameter.

When only the molecular weight of the particles has to be determined the measurement of the scattered light can be replaced by the observation of the attenuation of the primary beam passing through the sample. The *specific turbidity*, i.e. the ratio of the turbidity τ to the concentration, is proportional to the volume or the mass of the single particle so long as particles are so small that the scattering is uniform

* There is a proposal of KERKER (1952) for the use of unfiltered, polychromatic light in determining the mol. wt., but his method was hardly applied to any system.

over all angles. In this range the turbidity measurement which can be performed with any sensitive photometer, can substitute the more elaborate observation of the scattered light. As soon, however, as the angular dependence of the scattering can no longer be neglected, the turbidity decreases by a factor which depends on the shape of the particle and hence is not known in advance. In some special cases it can be deduced with sufficient precision from the dispersion of the turbidity and so the applicability of the method is extended to larger particles. But as a rule the uncertainty in this correction-factor sets a limit to the use of transmission measurements of the turbidity for molecular weight determination.

With very few exceptions the particles are not of uniform size and shape. Due to this rather inevitable *polydispersity* only an average volume or mass and gyration radius can be determined by light scattering. In some cases (random coils, spheres) also an estimate of the broadness of the polydispersity can be obtained. With other shapes, however, there are too many independent parameters influencing the scattering so that their contributions cannot be separated. As a matter of fact in such a case the influence of polydispersity cannot be clearly separated from that of shape.

Samples which can be investigated by the light scattering method may have very different compositions. The *particles* whose size and shape have to be determined can either have a very *porous structure* so that nearly all the bulk volume is filled by the solvent, or they may be *solid granules* or *liquid drops* definitely differing in their optical density from the solvent. Swollen proteins in good solvents are examples of the first case; suspensions, for instance polystyrene latex, and emulsions (oil in water) are representative of the second case. The porous particle has practically the same optical density as the pure solvent so that the polarizing field in it is the same as the field in the pure solvent. In the second case, however, the particle with higher (lower) polarizability than the solvent appreciably lowers (increases) the polarizing field as compared with that of the pure solvent, the effect being the larger the greater the difference in refractive index between the particle and the solvent.

The solvent can be either a one-component liquid (the solvent and the solute form a *two-component system*) or a mixture of liquids or a solution of low mol. wt. components (*many-component system*). The last case includes *polyelectrolyte* solutions where in most cases the solvent is water containing low molecular gegenions of the polyelectrolyte and the low molecular ions of the added salts. Such a system has at least 3 components. The charge on the particle and the ionic atmosphere surrounding the particle may strongly influence its size and shape and also its scattering contribution. By increasing the ionic strength of the

solution all those charge effects can be so far reduced that the size and shape of the particle becomes identical with that of the uncharged state. Soaps and detergents are a special case where micelle formation sets in at a critical monomer concentration c_0. The micelles can be treated as independent particles having the concentration $c - c_0$.

Since light scattering increases linearly with the weight or volume of the particles regardless of the forces keeping the particle together, it is an extremely useful method not only for the determination of the size and shape of single molecules and stable particles but also for investigating aggregation and association effects, micelle formation, the growth or dissolution of condensation nuclei in precipitating systems, etc. and the influence of different factors such as temperature, pH, salt concentration, solvent or non-solvent admixture, ageing, irradiation, etc. on these effects. The method is equally suitable for solutions, suspensions, emulsions and aerosols* under the condition that the particles do not change with increasing dilution.

The theory of light scattering is rather simple when the interaction effects of the optical field in different particles can be neglected. This is the case in *infinitely dilute solutions*. The experiments, however, are performed at finite concentration. Therefore the experimental data have first to be extrapolated to infinite dilution before they can be compared with theoretical calculations on the single *isolated particle*.

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III. Theory of Scattering

1. *Binary systems—Particles* (1) *in a one-component solvent* (2)

1.1. *Small particles* (*Rayleigh scattering*). As long as the particles in the sample are sufficiently small compared with the wave-length ($\lambda_0/n = \lambda$), i.e. smaller than $\lambda/10$, they can be substituted by a simple dipole

$$p = \alpha E \qquad \ldots.(1)$$

which gives the excess polarization over that of the solvent due to the particle in the optical field, $E = E_0 \cos \omega t$, of the incoming wave. The excess polarizability, α, depends on the shape and size of the particle. For spheres made of isotropic material it is

$$\alpha = \frac{3n_2^2}{4\pi} \cdot \frac{n_1^2 - n_2^2}{n_1^2 + 2n_2^2} V_1 = \frac{n_2^2}{4\pi} \cdot \frac{n_1^2 - n_2^2}{n_2^2 + \frac{1}{3}(n_1^2 - n_2^2)} V_1 \qquad \ldots.(2)$$

the induced dipole having the same direction as E. In the case of anisotropic particle material with three main values of the refractive

* In this short survey aerosols will not be treated.

index, $n_1^{(1)}$, $n_1^{(2)}$ and $n_1^{(3)}$, the polarizability of the sphere also becomes a tensor with the three main values

$$\alpha_j = \frac{3n_2^2}{4\pi} \cdot \frac{\{n_1^{(j)}\}^2 - n_2^2}{\{n_1^{(j)}\}^2 + 2n_2^2} V_1 \qquad j = 1, 2, 3 \qquad \ldots.(3)$$

the dipole being no longer parallel to E.

In the case of an ellipsoidal particle with the main axes, a_1, a_2 and a_3, and the main refractive indices, $n_1^{(1)}$, $n_1^{(2)}$ and $n_1^{(3)}$—as a rule the main axes of n coincide with the geometrical axes—one has

$$\alpha_j = \frac{n_2^2}{4\pi} \cdot \frac{\{n_1^{(j)}\}^2 - n_2^2}{n_2^2 + x_j(\{n_1^{(j)}\}^2 - n_2^2)} V_1 \quad j = 1, 2, 3 \qquad \ldots.(4)$$

where x_j are form factors depending on the ratio $a_1{:}a_2$ and $a_1{:}a_3$. Generally one considers rotational ellipsoids only, $a_2 = a_3$, $n_1^{(2)} = n_1^{(3)}$ for which case $4\pi x_1 = L_1$ and $4\pi x_2 = L_2$ are tabulated as functions of the ratio $f = a_1{:}a_2$ (PETERLIN and STUART, 1939)*.

The induced dipole oscillates with the same frequency as E and radiates energy which is observed as excess scattering over that of the pure solvent. For the sake of simplicity let us assume that the primary beam is horizontal (y-axis) and comes from the left, its electrical vector E being either vertical (index v), parallel to the z-axis, or horizontal (index h) parallel to the x-axis; or the light can be unpolarized (index u).

The average over all orientations of the particle yields for the scattered intensity (energy flux per cm^2) in the horizontal xy-plane at the angle θ between the observation direction and the positive y-axis:

$$I(\theta) = I_0(16\pi^4/r^2\lambda^4)\,\langle\alpha\rangle^2\psi(\theta) \qquad \ldots.(5)$$

where I_0 = intensity of the primary beam,
r = the distance of the observer from the sample,
$\langle\alpha\rangle = (\alpha_1 + \alpha_2 + \alpha_3)/3$ = the average excess polarizability of the particle,
$\psi(\theta)$ = the polarization factor depending on the polarization of the primary beam and the optical anisotropy of the particle.

Since the ratio $I(\theta)/\psi(\theta)$ is independent of the angle the scattering of the single dipole is called *isotropic scattering*. When all the particles are identical and completely independent, their scattering intensity has simply to be summed up to give the scattering of the sample. With N_1

* The optical anisotropy due to the difference in the factors L_j ($L_{\text{sphere}} = 4\pi/3$) is usually called *form* or *shape anisotropy*, and that due to the difference in $n_1^{(j)}$ is the *eigen* or *intrinsic anisotropy*.

particles per ml the scattering of the volume V of the sample uniformly illuminated by the primary beam equals

$$I_V(\theta) = VN_1I(\theta) = I_0Vc(16\pi^4M/r^2\lambda^4N)\langle\alpha\rangle^2\psi(\theta) \quad \ldots.(6)$$

where N = Avogadro number,
M = molecular weight of the particle,
$c = N_1M/N$ = concentration in grammes of the solute per ml of the solution.

The average excess polarizability is closely connected with the *refractive index increment* of the solution

$$\frac{n^2 - n_2^2}{n^2 + 2n_2^2} = \frac{4\pi}{3} N_1\langle\alpha\rangle \quad \ldots.(7)$$

at concentrations so low that the interaction effects among the particles can be completely neglected. In this case equation (7) yields

$$\langle\alpha\rangle = (M/2\pi Nn)\,(\mathrm{d}n/\mathrm{d}c) \quad \ldots.(8)$$

and one obtains from equation (6) the *reduced intensity* as

$$R(\theta) = r^2I_V(\theta)/I_0V = c(4\pi^2Mn^2/N\lambda_0^4)\,(\mathrm{d}n/\mathrm{d}c)^2\psi(\theta) \quad \ldots.(9)$$
$$= cKM\psi(\theta)$$

where $K = (4\pi^2n^2/N\lambda_0^2)\,(\mathrm{d}n/\mathrm{d}c)^2$

For *isotropic particles*, i.e. for spheres made from isotropic material, the polarization factor is (Fig. 2)

$$\psi_v = 1; \quad \psi_h = \cos^2\theta; \quad \psi_u = (1 + \cos^2\theta)/2 \quad \ldots.(10)$$

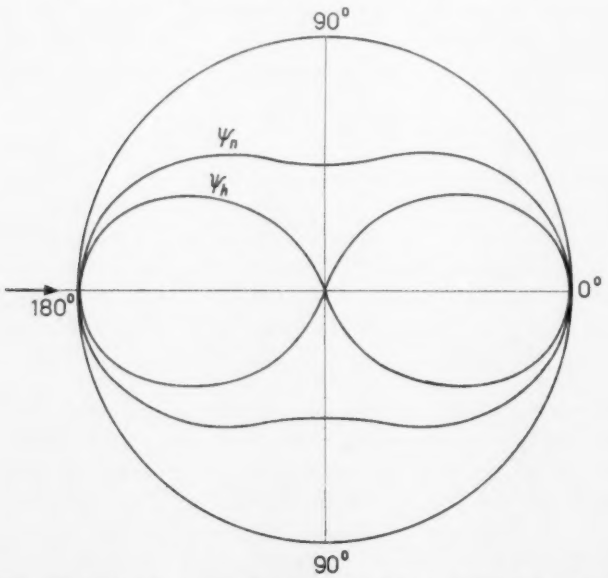

Fig. 2. The polarization factors ψ_v (circle), ψ_h and ψ_u for small isotropic particles.

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At $\theta = 0$ one has $\psi = 1$. The scattered light at $\theta = 0$ has the same polarization as the primary beam and is linearly polarized at $\theta = 90°$ with

$$\psi_v = 1; \quad \psi_h = 0; \quad \psi_u = 1/2 \qquad \ldots.(11)$$

Anisotropic particles, i.e. ellipsoids (rods, disks), spheres and coils made from anisotropic material, yield a more complicated behaviour. As a main characteristic the polarization of the scattered light changes as compared with that of isotropic spheres. At $\theta = 90°$ the horizontal components of ψ do not vanish but have finite values

$$H_v = H_h = H_u \neq 0 \qquad \ldots.(12)$$

and the ratio of the vertical components changes

$$V_v \neq V_h \neq 0; \quad V_u = (V_v + V_h)/2 \qquad \ldots.(13)$$

Mostly the *depolarization ratios* are measured

$$\Delta_v = H_v/V_v; \quad \Delta_h = H_h/V_h = 1;$$

$$\Delta_u = H_u/V_u = (H_v + H_h)/(V_v + V_h) = 2\Delta_v/(1 + \Delta_v) \qquad \ldots.(14)$$

Both quantities are closely connected with the *optical anisotropy* δ^2 of the particle

$$\delta^2 = \frac{(\alpha_1 - \alpha_2)^2 + (\alpha_2 - \alpha_3)^2 + (\alpha_3 - \alpha_1)^2}{(\alpha_1 + \alpha_2 + \alpha_3)^2} = \frac{10\Delta_u}{6 - 7\Delta_u} \qquad \ldots.(15)$$

The upper limits for δ^2, Δ_u and Δ_v are 2, $\frac{1}{2}$ and $\frac{1}{3}$ respectively. In most cases they are much lower, e.g. δ^2 is 0·12 for the $(CN)_2$ molecule and drops to a few hundredths for nitrocellulose or polystyrene of medium mol. wt. ($M = 100{,}000$). At $\theta = 0$, however, one has independently of particle size

$$\Delta_u(0) = 1 \qquad \Delta_v(0) = 1/\Delta_h(0)$$

$$\delta^2 = \frac{10\Delta_v(0)}{3 - 4\Delta_v(0)} \qquad \ldots.(16)$$

The depolarization as measured by Δ_v or Δ_u at infinite dilution is a sure indication of the optical anisotropy of the particle. The most important consequence is the modification of $\psi(0)$ to

$$\psi_u(0) = 1 + 2\delta^2/5 = (6 - 3\Delta_u)/(6 - 7\Delta_u) \qquad \ldots.(17)$$

the correction usually being a few per cent (CABANNES, 1929).

As a matter of fact, the light scattered by low molecular weight solvents (benzene, toluene, acetone, etc.) is depolarized to a very high degree (see Table 1), thus making less easy the detection of low depolarization ratios of the excess scattering of the investigated particles.

All the light scattered by the particles is taken away from the primary beam. Its intensity hence decreases exponentially*

$$I_0(x) = I_0(0)\, e^{-\tau x} \qquad \ldots.(18)$$

the *turbidity*, τ being given by the total amount of scattering by the particles in 1 ml in all directions

$$\tau = \frac{1}{I_0}\int I_{ml}(\theta) r^2\, d\Omega = c(4\pi^2 n^2 M/N\lambda_0^4)\,(dn/dc)^2 \int \psi(\theta)\, d\Omega$$

$$= cHMQ \qquad \ldots.(19)$$

where $$H = (32\pi^3 n^2/N\lambda_0^4)\,(dn/dc)^2 = (8\pi/3)K$$

$$Q = (3/8\pi)\int \psi(\theta)\, d\Omega$$

The factor Q depends on the angular variation of the scattering:

$$Q = 1, \qquad \text{for isotropic particles}$$

$$= 1 + \delta^2 = (6 + 3\Delta_u)/(6 - 7\Delta_u), \text{ for anisotropic particles} \ldots.(20)$$

The correction due to the anisotropy of the particle is rather large, two and half times larger than in $\psi_u(0)$, and hence often cannot be neglected.

Because of the proportionality between the turbidity and the reduced scattering intensity in the case of small particles

$$\tau = (8\pi/3)QR(\theta)/\psi(\theta) \qquad \ldots.(21)$$

very often the latter quantity is simply called turbidity although it was obtained from scattering at a fixed angle and not from the attenuation of the primary beam.

The mol. wt. may be obtained either from equation (19):

$$M = \tau/cH, \qquad \text{for isotropic particles}$$

$$= (\tau/cH)(6 - 7\Delta_u)/(6 + 3\Delta_u), \text{ for anisotropic particles} \quad \ldots.(22)$$

irrespective of the polarization of the light beam, or from equation (9):

$$M = R(\theta)/cK\psi(\theta) \qquad \ldots.(23)$$

The constant ratio $R(\theta)/\psi(\theta)$ is very often measured at a rather high angle (back scattering) because as a rule in that case the contribution of the mainly forward scattering by dust particles which remained in

* It is assumed that the extinction in pure solvent can be neglected. If this is not so the difference of the extinction coefficient between solution τ_s and solvent τ_2, i.e. the *excess turbidity*

$$\tau = \tau_s - \tau_2$$

has to be introduced in equation (19).

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the sample in spite of thorough purification, can be completely neglected.

By extrapolating the measurements at higher angles to $\theta = 0$ one obtains:

$$M = R(0)/cK, \qquad \text{for isotropic particles}$$

$$= [R(0)/cK](6 - 7\Delta_u)/(6 - 3\Delta_u), \text{ for anisotropic particles} \quad \ldots.(24)$$

irrespective of the size and shape of the particles. This method is mainly applied in the case of particles of large molecular weight. It only requires measurements at angles so low that an unambiguous extrapolation to $\theta = 0$ can be performed. This procedure becomes difficult with very monodisperse suspensions of large spheres where the scattered intensity oscillates very rapidly even at small angles.

With small particles one prefers to measure at 90°. In this case we have for isotropic particles:

$$M = 2R(90°)/cK, \text{ for unpolarized light}$$

$$M = R(90°)/cK, \quad \text{for vertically polarized light} \quad \ldots.(25)$$

and for anisotropic particles:

$$M = [2R(90°)/cK](6 - 7\Delta_u)/(6 + 6\Delta_u), \text{ for unpolarized light}$$

$$M = [R(90°)/cK](3 - 4\Delta_v)/(3 + 3\Delta_v), \quad \text{for vertically polarized light} \quad \ldots.(26)$$

Of course the corrections due to the depolarization have to be taken into account only if they are larger or of the same order of magnitude as the experimental error in R and K. Special attention is needed in the measurement of refractive index increment whose square enters H and K.*

As examples of very low mol. wts. which can still be determined by light scattering we have to mention the experiments of Strauss *et al.* (1953) on sodium polyphosphate in 0·1 to 0·35 N sodium bromide solutions with M between 7400 and 18,200; those of Price *et al.* (1956) on polysiloxanes in precipitant solvents (ideal solutions) with M as low as 1000; of Breitenbach (1956) on polystyrene in toluene with M between 8000 and 19,000; of Mathews (1956) on sodium chondroitin sulphate in 0·2 N sodium chloride solution with M between 18,000 and

* There is still much discrepancy in experimental results on dn/dc among different investigators as can be seen from the mol. wt. measurement reports of standard polystyrene samples (Frank and Mark, 1953, 1955) and from the compilation of data on polymethylmethacrylate by Jose and Biswas (1957). "Anomalous" mol. wts. of PMMA in butanone as reported by Bhatnagar and Biswas (1954) were mainly due to such an error in the rather low dn/dc of this system as it was shown by Cohn and Schnele (1954) and Tremblay *et al.* (1954).

150,000; of KATCHALSKY *et al.* (1957) on polyvinylpyridine in chloroform and polyvinylpiperidine in methanol with M from 10,000 to 670,000; of YASNOFF and BULL (1953), KRONMAN and STERN (1955) on pepsin with M about 35,000 and of GUINAND and JOLIOT (1957) on trypsin with $M = 25{,}000$.

With increasing mol. wt. the scattering rapidly increases, making the method easier to apply. It is therefore now one of the most widely used techniques for that purpose and there would be little sense in enumerating here the long series of papers dealing with the subject.

Polydispersity. As a rule the particles of a colloidal solution have different sizes and often also different shapes. Hence the mol. wt. determined from light scattering or turbidity is only an average over all sizes. When particles with the mol. wt. M_j have the partial concentration c_j, than one deduces from equation (9)

$$R(\theta) = K\sum_{(j)} c_j M_j \psi_j(\theta) = cKM_R\psi(\theta)$$

$$\tau = H\sum_{(j)} c_j M_j Q_j = cHM_\tau Q \qquad \ldots.(27)$$

The average mol. wts. M_R and M_τ still depend on the average polarization factors ψ and Q respectively which are not known in advance:

$$M_R = R(\theta)/cK\psi(\theta)$$

$$M_\tau = \tau/cHQ \qquad \ldots.(28)$$

Such a case is realized in a polydisperse solution of randomly coiled linear macromolecules of a moderate degree of polymerization. In such molecules the optical anisotropy δ^2 varies rather strongly with the mol. wt. (WEILL, 1958.)

When the particles are similar, i.e. if they have all the same shape and the same anisotropy, for instance, if they are homogeneous spheres differing in the radius only, then these factors are the same for all particles and M from equation (27) becomes the *weight average*:

$$M_w = \sum c_j M_j/c = R(\theta)/cK\psi(\theta) = \tau/cHQ \qquad \ldots.(29)$$

In the weight average the influence of large particles prevails over that of the small ones, so that even large admixtures of small particles are not easily detectable by light scattering. On the contrary it is very easy to detect the presence of large particles, for instance their growth by association. In this way, for instance, DOTY *et al.* (1947) have investigated the association of polyvinylchloride in dioxane as a function of temperature and concentration and the association of some other polymers in poor solvents close to the phase-separation; EDELHOCH *et al.* (1953) the dimerization of serum mercaptalbumin in presence of mercurials; BLUM (1955), TONOMURA (1956), TONOMURA

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et al. (1957a b c) the interaction of serum triphosphate and natural actomyosin; TEŽAK *et al.* (1951) the coagulation of silver halide sols *in statu nascendi*; BHATTACHARYA and KUMAR (1951), KUMAR and BHATTACHARYA (1951, 1952a b), BHATTACHARYA (1956) that of arsenious sulphide, chromium hydroxide and ferric hydroxide and SIVARAJAN (1955) that of silver sols.

1.2. *Large particles* (*Mie-scattering*). When the particles are not negligibly small as compared with the wave-length in the medium, the single dipole representing the excess polarizability of the particle has to be replaced by an infinite series of electric and magnetic multipoles of increasing order which are chosen in such a way that the boundary conditions for the optical field on the surface of the particle are fulfilled. The problem was solved by MIE (1908) for spherical transparent and absorbing particles of any radius and there are also some less general solutions and approximations for ellipsoids (MÖGLICH, 1927; JONES, 1945; SCHULTZ, 1950; TAI, 1952; STEVENSON, 1953) and for cylinders (BURBERG, 1956).

From the Mie solution extensive tables* of light-scattering functions for spheres of radii from some hundred to many ten thousand Å and of different relative refractive indices $m = n_1/n_2$ were prepared which greatly facilitate the evaluation of experimental data and especially

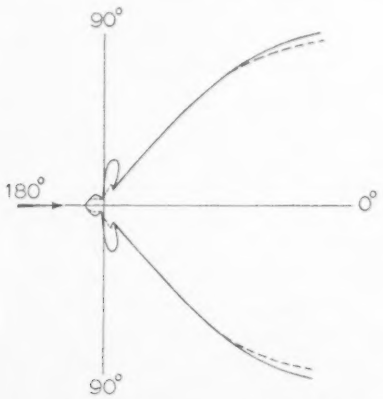

Fig. 3. The angular dependence of the light scattered by a large sphere with $J = 4$, $m = 1{\cdot}25$. The full line gives the intensity of V_u, the broken line that of H_u (from BLUMER, 1926).

* See the list of the existing tables by KERKER (1955). Most of them treat particles with rather high index of refraction (the complex values are for absorbing particles) as it occurs in aerosols. In the suspensions of organic particles the relative index m is much lower, mainly less than 1·2. This range is extensively covered by the new tables of PANGONIS *et al.*, (1957) and in part also by those of BLUMER (1925, 1926), GUMPRECHT and SLIEPCHEVICH (1951, 1953). VAN DE HULST (1946), and SINCLAIR and LA MER (1949) have given curves which permit interpolation between different values of α to obtain the size and concentration from transmission data.

permit the determination of the particle radius from the observed angle dependence and dispersion of the scattered light or from the turbidity of the sample. Due to the large size of the particles the scattering is very intense making the measurement rather easy.

With increasing $\alpha = 2\pi rn/\lambda_0$ the angle dependence becomes very strong (Fig. 3). At $\alpha > \pi/2$ the intensity oscillates and rapidly decreases with increasing θ. As a consequence of the *dispersion* the scattering of white light shows very easily observable colour changes (higher order Tyndall spectra) with increasing θ which effect was used by JOHNSON and LA MER (1947), LA MER (1949), LA MER and PLESNER (1957) and MEEHAN (1958) for rapid determination of radii of very uniform sulphur hydrosols, oil aerosols and aqueous polystyrene latex suspensions. Further the simple λ^{-4} scattering law is the more infringed the higher α. As a consequence the colour of scattered light changes from blue to white. The depolarization at 90° does not vanish the more as it does in the case of small isotropic spheres. Hence also from the observed *depolarization* the size of spheres can be determined (JOHNSON and LA MER 1947, KERKER and LA MER 1950). It is worth while to note that in determining the radius from the angular dependence of scattered light the knowledge of absolute concentration is not wanted, one has only to extrapolate to infinite dilution in order to get rid of interaction effects.

The polydispersity of the samples the more complicates the evaluation of light scattering data the bigger the particles. The maxima and minima of the angular scattering function are flattened and dislocated, the distortion being strongly dependent on the actual size distribution of the sample. Among others ATHERTON and PETERS (1953a), CHIN *et al.* (1955) have developed evaluation methods for such cases. It has to be noted that with bigger particles the radii determined are not simple weight averages.

The *specific turbidity* τ/c measurements represent a very simple and rapid method for radii determination of monodisperse sphere suspensions (LA MER and BARNES 1946, BARNES and LA MER 1946). STRATTON and HOUGHTON (1931) and BRILLOUIN (1949) give extinction curves for particles with diameters up to 50 μ. A rough test on polystyrene latex, glass spheres and sand suspensions was given by ROSE (1950a b) who proposes a modification and extension of the theoretical curve for determination of size characteristics of fine powders (ROSE 1951). ATHERTON and PETERS (1952) find a good agreement in size of cellosolve methacrylate from angular dissymmetry and turbidity under the assumption that the particles are colourless isotropic spheres. TABIBIAN *et al.* (1956), HELLER and TABIBIAN (1956), HELLER (1957a b) have worked out exact graphs (Fig. 4) and simple interpolation formulae which with a sufficient accuracy (better than 5 per cent)

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reproduce the Mie-values for spheres with α from 0·8 to more than 6 and m between 1 and 1·25. For instance the expression

$$\log(\lambda\tau/c') = 4080 - 1060/\alpha + (2084 - 0{\cdot}0564/\alpha)\log(m - 1) \quad \ldots.(30)$$

with c' volume of solute per ml of the solution, is proposed for $0{\cdot}8 \leqslant \alpha \leqslant 2{\cdot}0$ and $1{\cdot}0 \leqslant m \leqslant 1{\cdot}30$.

As is evident from Fig. 4 one measurement of τ/c at known m does not give an unambiguous value for the radius. One has to measure at

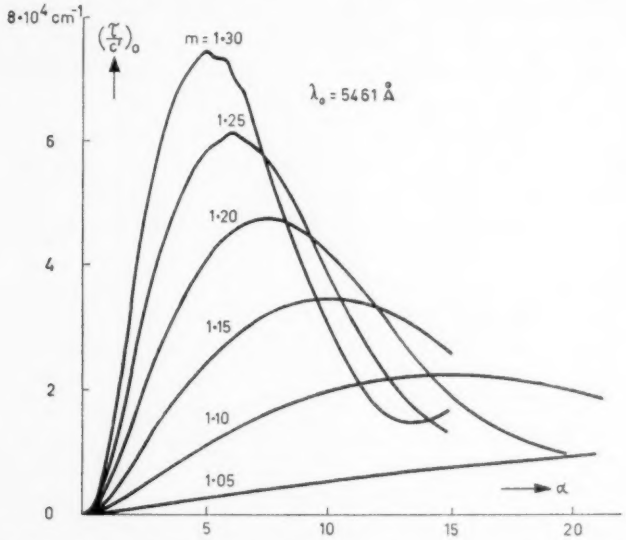

Fig. 4. Specific turbidity (limiting value of the ratio of the turbidity by the volume concentration c' at infinite dilution) of a suspension of spheres of different relative index of refraction $m = n_1/n_0$ and $\alpha = 2\pi r\, n_0/\lambda_0$ (from HELLER and PANGONIS, 1957).

different wave-lengths and compare the slope $\mathrm{d}(\tau/c)/\mathrm{d}(1/\lambda)$ with those of the pertinent m-curve at all points with the same ordinate $(\tau/c)_{\mathrm{obs}}$. The point with the best fit will yield the correct α of the particles. The polydispersity complicates the evaluation of light scattering data. The maxima and minima of the specific turbidity are flattened and dislocated. Among others ATHERTON and PETERS (1953a), CHIN *et al.* (1955) have developed evaluation methods for such cases.

BILLMEYER (1954) has proposed an extrapolation method to infinite wave-length, where the usual small particle light scattering treatment is valid. He plots Hc/τ over $(n/\lambda_0)^2$ and obtains straight lines for polystyrene latex suspensions with $\alpha(m - 1) = 0{\cdot}2$–$0{\cdot}6$. The intercept of the ordinate yields the mol. wt. of the particles from which the radius is easily obtained. The method is applicable for particles with

$\alpha(m-1) < 2$. In the case of a polydisperse sample it yields the weight average of M and r^3.

For non-spherical particles there are only very limited theoretical data and tables (SCHULTZ, 1950; TAI, 1952) which can hardly cover the practical needs in the determination of particle size and shape by light scattering or turbidity measurements. But since there are at least two parameters (volume and the axial ratio of the rotational ellipsoid) instead of one for spherical particles (the radius of the sphere) and since the samples show a polydispersity in both of them, the situation is very likely so complicated that one cannot hope that light scattering could give enough information for the determination of the size and shape of the non-spherical particles, and, moreover, very likely the method would not be very simple. Measurement of vanadium pentoxide hydrosols (KERKER *et al.*, 1954), which were also investigated by electron microscopy, cannot yet be interpreted in terms of the existing theories. The situation in this case is still more complicated by the true absorption of light in the particles and by the partial dissolution of the particles at dilution of the sol.

1.3. *Large particles with relative index of refraction $m = n_1/n_2$ very close to unity.* For such particles the influencing electrical field inside the particle can be assumed to be the same as in the free solvent (DEBYE, 1947). Such a situation is found in a suspension of porous particles, e.g. swollen proteins, where the main part of the bulk volume occupied by the particle is filled with the solvent or in a suspension where by proper choice of the solvent its optical density has been nearly equalled with that of the impermeable particle. The closer $m = n_1/n_2$ is to unity the better the approximation in the internal field and the larger particle dimensions to which it can be extended.

In addition all coiled linear macromolecules are also treated as if the difference in the refractive index between the solvent and solute were negligibly small. Actually, due to the rather low density inside the coil the mean electrical field in the coil does not differ markedly from that in the pure solvent.

In such a case in every volume element $\mathrm{d}V$ of the particle an excess dipole moment

$$\mathrm{d}p = \frac{1}{4\pi}(n_1^2 - n_2^2)\, E\, \mathrm{d}V \qquad \ldots.(31)$$

is induced, oscillating with the same frequency as the field vector E of the linearly polarized incoming light. The square of the sum of the field vectors of all the light waves emitted by the single induced elementary dipoles yields the intensity of the light scattered by the particle. For random orientation one obtains the reduced intensity

$$R(\theta) = cKM\psi(\theta)\,.\,P(\theta) \qquad \ldots.(32)$$

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The interference function $P(\theta)$, measuring the reduction of the scattered intensity due to the phase differences between the single elementary dipoles in the particle, depends strongly on the special shape of the particle.

Spheres (RAYLEIGH, 1914), e.g. globular proteins

$$P(\theta) = \left[\frac{3}{x^3}(\sin x - x\cos x)\right]^2 = 1 - \frac{x^2}{5} + \frac{3x^4}{25} - \ldots$$

$$= 1 - \frac{1}{5}\left(\frac{4\pi nr}{\lambda_0}\right)^2 \sin^2\frac{\theta}{2} + \ldots \quad \ldots.(33)$$

where $x = (4\pi nr/\lambda_0)\sin(\theta/2)$

$$P^{-1}(\theta) = 1 + x^2/5 + 4x^4/175 + 47x^6/23{,}625 + \ldots$$

There are also formulae for ellipsoids* (ROESS and SHULL, 1947; POROD, 1948a b; DEBYE and ANNACKER, 1950; FOURNET and GUINIER, 1950) which in most cases were first derived for X-ray scattering. According to POROD (1948a b) the asymptotic decrease of P is proportional to $1/\sin(\theta/2)$ for elongated rod-like and to $1/\sin^2(\theta/2)$ for oblate disk-like ellipsoids. Hence by plotting $[R(\theta)/\psi(\theta)]\sin^2(\theta/2)$ over $\sin(\theta/2)$ one obtains a horizontal asymptote in the second and an asymptote with finite slope in the first case.

Very thin rods† (NEUGEBAUER, 1943), e.g. tobacco mosaic virus,

$$P(\theta) = \frac{1}{y}\int_0^{2y}\frac{\sin t}{t}\,dt - \left(\frac{\sin y}{y}\right)^2 = 1 - \frac{y^2}{9} + \frac{2y^4}{225} - \ldots$$

$$= 1 - \left(\frac{2\pi nl}{3\lambda_0}\right)^2 \sin^2\frac{\theta}{2} + \ldots \quad \ldots.(34)$$

where $y = (2\pi nl/\lambda_0)\sin(\theta/2)$

$$P^{-1}(\theta) = 1 + y^2/9 + 7y^4/2025 + \ldots$$

l = length of the rod $\gg d$ = diameter of the rod.

Random coil (DEBYE, 1944), e.g. the statistically coiled linear, unbranched macromolecule,‡

$$P(\theta) = \frac{2}{z^2}(z - 1 + e^{-z}) = 1 - \frac{z}{3} + \frac{z^2}{12} - \ldots$$

$$= 1 - 2\left(\frac{2\pi nR}{3\lambda_0}\right)^2 \sin^2\frac{\theta}{2} + \ldots \quad \ldots.(35)$$

* See the excellent review by EDSALL and DANDLIKER (1951).

† See also STOKES (1957).

‡ HERMANS (1958) has also calculated P for the random coil with non-negligible segment length (zig-zag chain consisting of Z rods of length A).

where

$$z = (8\pi^2 n^2 R^2/3\lambda_0^2) \sin^2 (\theta/2)$$

$$P^{-1}(\theta) = 1 + z/3 + z^2/36 + \ldots$$

R^2 = mean square of the end-to-end distance of the coil.

Coil with persistence length a and extended length $L = p \cdot a^*$ (PETERLIN, 1953b c), e.g. desoxyribonucleic acid,

$$P_p(\theta) = e^u[F(u,p) - (u/1!)F(u+1,p) + (u^2/2!)F(u+2,p) - \ldots]$$

$$= 1 - \frac{up}{3}\left(1 - \frac{p}{5} + \frac{p^2}{30} - \ldots\right) + \ldots$$

$$= 1 - \left(\frac{4\pi na}{\lambda_0}\right)^2 \frac{L}{3a}\left(1 - \frac{L}{5a} + \ldots\right) \sin^2 \frac{\theta}{2} + \ldots \qquad \ldots.(36)$$

where

$$u = (16\pi^2 n^2 a^2/3\lambda_0^2) \sin^2 (\theta/2)$$

$$F(u,p) = [2/(up)^2]\,(up - 1 + e^{-up})$$

At $a = \infty$ such a coil is fully stretched and becomes a rod with $L = l$, at $a = 0$, however it approaches the random coil.

In Fig. 5 the functions P^{-1} for all the four cases are plotted against v

$$v = t \sin^2 (\theta/2) \qquad \ldots.(37)$$

where t is chosen in such a way that all the curves have the same slope at the origin. The value of t is given by the coefficient of the first term in $\sin^2 (\theta/2)$ in the series expansion of P in equations (33), (34), (35), and (36). Since the coefficients of the series expansion of P in powers of $\sin^2 (\theta/2)$ contain mean values of the increasing moments of the intramolecular distances

$$P(\theta) = 1 - \frac{8\pi^2 n^2}{3\lambda_0^2}\langle r_{ij}^2\rangle \sin^2 (\theta/2) + \frac{32\pi^4 n^4}{15\lambda_0^4}\langle r_{ij}^4\rangle \sin^4 (\theta/2) - \ldots$$

$\ldots.(38)$

and $\langle r_{ij}^2\rangle$ is just twice the square of the so called *gyration radius*,† ρ, (ZIMM and STOCKMAYER, 1949), one has

$$P^{-1}(\theta) = 1 + \frac{16\pi^2 n^2}{3\lambda_0^2}\rho^2 \sin^2 \frac{\theta}{2} + \ldots = 1 + v + \ldots \qquad \ldots.(39)$$

with

$$t = 16\pi^2 n^2 \rho^2/3\lambda_0^2 \qquad \ldots.(40)$$

* The persistence length (KRATKY and POROD, 1949) measures the orientation of subsequent chain elements. In sufficiently long chains it equals half the length A_m of the Kuhn's preferential statistical chain segment. In that case the mean square of the end-to-end distance R^2 is $2aL = ZA_m{}^2$, $L = ZA_m$. Generally one has $R^2 = 2a^2(p - 1 + e^{-p})$.

† The gyration radius is the mean value of the square of the distance between the centre of the mass and any mass element of the particle.

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The curves P^{-1} in Fig. 5 correspond to particles with the same gyration radius. From equations (33), (34) and (35) one deduces:

$$2r = 2{\cdot}58\rho, \text{ for a sphere}$$

$$l = 3{\cdot}46\rho, \text{ for a rod}$$

$$R = 2{\cdot}45\rho, \text{ for a random coil*} \qquad \ldots.(41)$$

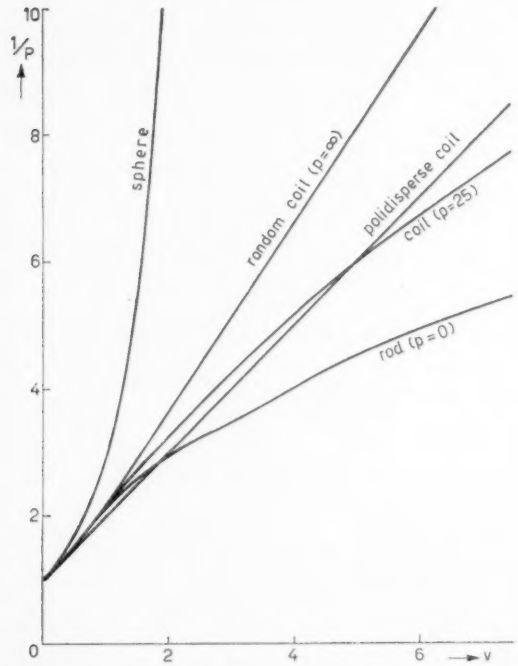

Fig. 5. P^{-1} against v (equations 37 and 40) for spheres, random coils ($p = \infty$), coils with persistence length $a = L/25$, rods, and for polydisperse random coils ($p = \infty$) with the special molecular weight distribution as given by equation 46 (Fig. 9).

For the coil with finite persistence length the ratio R/ρ increases from 2·45 at $p = \infty$ to 3·46 at $p = 0$.

The gyration radius of the particle is obtained from the slope t of the experimentally determined P or P^{-1} plotted *versus* $\sin^2(\theta/2)$,

$$\rho^2 = 3\lambda_0^2 t/16\pi^2 n^2 \qquad \ldots.(42)$$

Its determination is independent of any assumption concerning the shape of the particle. In principle, also, higher moments of the

* Linear macromolecules in good solvents are more extended than the random coil. The square of their diameter increases as $M^{1+\varepsilon}$ instead of M as required by the random coil model. In this case one has very nearly $R = (2{\cdot}45 + \varepsilon)\rho$ or better $R^2 = (2 + \varepsilon)(3 + \varepsilon)\rho^2$ (Peterlin, 1955b). The calculation of P for such coils was performed by Ptitzyn (1957), Benoit (1957) and Loucheux *et al.* (1958).

intramolecular distances can be determined from P and from them the shape of the particle deduced. But due to the rather low precision of the measurements and to the unavoidable polydispersity of the samples this procedure cannot be successfully applied. In normal practice the experimental P^{-1} curve plotted against $t \,.\, \sin^2(\theta/2)$ is compared with Fig. 5 and from the best fit with one of the theoretical curves the corresponding shape of the particles deduced (Fig. 12). In most cases, however, the shape, viz. sphere, rod or coil, is already known in advance. Then the deviations from the theoretical curve can be interpreted as a

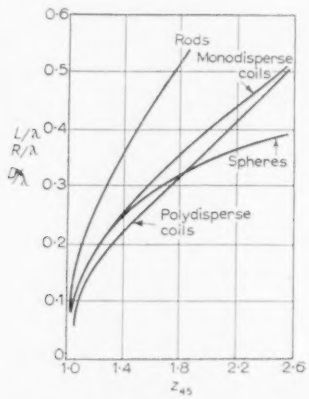

Fig. 6. Particle dimensions; L/λ (rods), R/λ (coils), D/λ (spheres) with $\lambda = \lambda_0/n_n$ as function of the dissymmetry $z_{45} = R_{45}/R_{135}$ (from Doty and Steiner, 1950). The polydisperse coils correspond to the molecular weight distribution function of equation 46 (Fig. 9).

consequence of the polydispersity or of some specific deviations of the particle shape and material from the model, e.g. the persistence in the random coil model (Peterlin, 1953b c), non-Gaussian character of the coil (Benoit and Doty, 1953), branching of the linear macromolecule (Benoit, 1955) or optical anisotropy of the particle material (Horn *et al.*, 1951). But it is impossible to separate the influence of all these causes, so that only one of them can be estimated when the influence of the others can be excluded.

Dissymmetry of the scattered light. Instead of measuring the whole angle dependence of the scattering and extrapolating to $\theta = 0$, one often prefers to measure at two angles symmetrical to 90°. Mainly one measures at 45° and 135°. The ratio $z_{45} = R_{45}/R_{135}$ extrapolated to infinite dilution (*intrinsic dissymmetry coefficient*) is 1 for very small particles and increases with the dimensions of the particle. It can be easily calculated from P and vice versa the characteristic dimension read from a plot of z_{45} (Fig. 6). The method has the great advantage

that the polarization factors and also nearly all other corrections for absorption, scattering, volume and refractive index just cancel out or are substantially reduced. This fact explains the wide use of this technique. But it has the disadvantage that only well defined particle shapes can be investigated. As soon as polydispersity or deviation of the shape from that of the model (stiffness, non-Gaussian chain, branching) markedly influence the angular dependence the method can no longer be applied or it yields the more incorrect results the larger the particles.

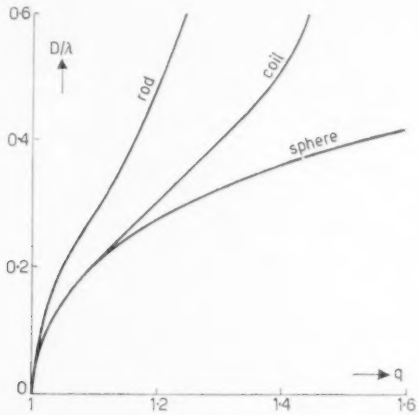

Fig. 7. Particle dimensions: L/λ (rods), R/λ (coils), D/λ (spheres) with $\lambda = \lambda_1/n_0'$ as function of the dispersion $q = \lambda_2^4 R(\lambda_1)/\lambda_1^4 R(\lambda_2)$ with $\lambda_1 = 4358$ Å, $\lambda_2 = 5461$ Å and $n_0' = 1{\cdot}389$, $n_0'' = 1{\cdot}381$ (MEK; 25°C).

Dispersion of the scattering also gives enough information for the determination of the dimensions of the particle. One usually measures at 90° at two wave-lengths, e.g. 4358 and 5461 Å, to determine the ratio

$$q = \lambda_2^4 R(\lambda_1)/\lambda_1^4 R(\lambda_2) \qquad \ldots.(43)$$

and reads the dimension of the particle of known shape from the pertinent plots of $q = P(\lambda_1/n_0')/P(\lambda_2/n_0'')$ where n_0' and n_0'' are the refractive indices of the solvent at λ_1 and λ_2 respectively (Fig. 7). This very simple method is less advantageous than the dissymmetry method because the corrections for depolarization, absorption and refractive index only partly cancel out.

Dispersion of turbidity also yields the particle dimensions when the correction factor Q from equation 19 is known. According to Doty and Steiner (1950) one measures the turbidity at two wave-lengths λ_1 and λ_2 and determines the quantity

$$\beta = \mathrm{d}\log(\tau\lambda)/\mathrm{d}\log\lambda = \mathrm{d}\log Q/\mathrm{d}\log\lambda \qquad \ldots.(44)$$

with $\lambda = \lambda_0/n$. The characteristic dimension L/λ (rods), R/λ (coils), D/λ (spheres) is then obtained from the inverse plot of $d \log Q/d \log \lambda$ over L/λ, R/λ or D/λ respectively (Fig. 8).

Both methods, however, have in common all the above mentioned disadvantages of the dissymmetry method.

Polydispersity. The turbidity and the extrapolated reduced intensity $R(0)$ yield the weight average mol. wt. M_w, the initial slope α_0 of the

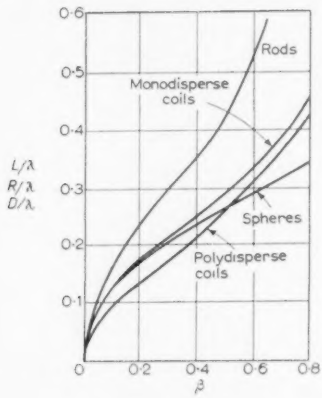

Fig. 8. Particle dimensions; L/λ (rods), R/λ (coils), D/λ (spheres) with $\lambda = \lambda_0/n_0$ as functions of the logarithmic dispersion of the turbidity: $\beta = \mathrm{d} \log (\tau\lambda^4)/\mathrm{d} \log \lambda$ (from DOTY and STEINER, 1950). The polydisperse coils correspond to the molecular weight distribution function of equation 43 (Fig. 9).

$cK\psi(\theta)/R(\theta)$ curve against $\sin^2(\theta/2)$ the z-average of the gyration radius (ZIMM and DOTY, 1944)

$$\rho_z^2 = \Sigma c_i M_i \rho_i^2 / \Sigma c_i M_i = 3 M_w \lambda_0 \tan \alpha_0 / 16\pi^2 n^2 \qquad \ldots.(45)$$

For the special case of the randomly coiled macromolecules with vanishing persistence length ZIMM (1948) and DOTY and STEINER (1950) have calculated the P^{-1} values for different degrees of polydispersity. For the special distribution function (Fig. 9)

$$f(M) = (4M/M_w^2) \exp(-2M/M_w) \qquad \ldots.(46)$$

yielding for the ratio of the averages $M_n : M_w : M_z$ the often found value 1:2:3, the pertinent P^{-1}, Z_{45}, q, Q are plotted in Figs. 5, 6, 7, 8. The function P^{-1} against $\sin^2 \theta/2$ is represented by a straight line. BENOIT (1953), BENOIT *et al.* (1954) point out that the reciprocal of the double ordinate intercept of the asymptote to the $cK\psi/R$ curve gives the number average mol. wt. M_n (Fig. 10).

$$M_n = 1/(2 \times \text{intercept of asymptote}) \qquad \ldots.(47)$$

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At the mol. wt. distribution yielding the averages M_n, M_w and M_z in the ratio 1:2:3 the experimental curve $cK\psi/R$ is a straight line. At higher polydispersity it shows a downward curvature roughly similar

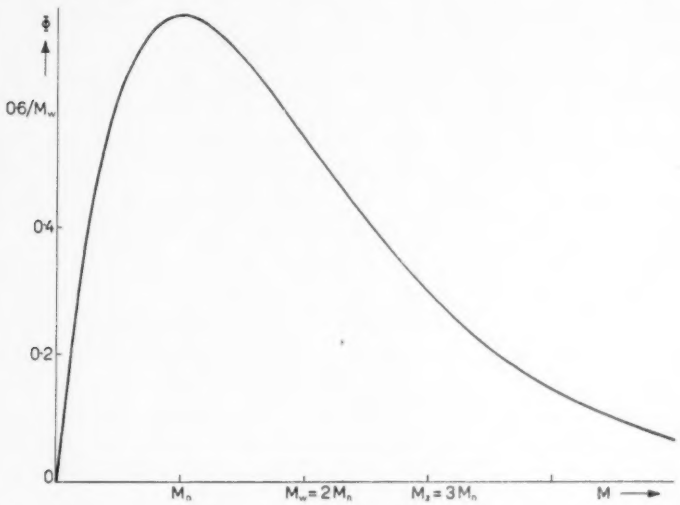

Fig. 9. Molecular weight distribution function $\phi(M) = 4M/M_w^2 \exp(-2M/M_w)$. In the interval between M and $M + dM$ one has $dN_1 = N_1 M_n \phi \, dM/M$ molecules. N_1 is the number of molecules per ml.

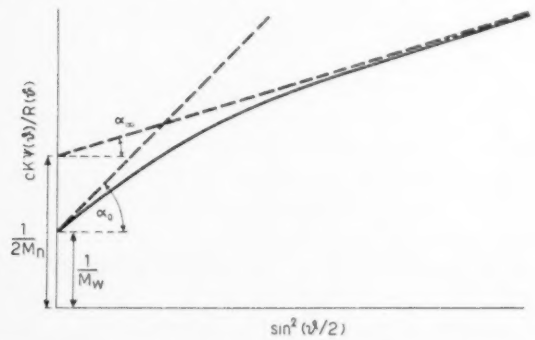

Fig. 10. The inverse scattering envelope $cK\psi(\theta)/R(\theta)$ over $\sin^2 \theta/2$ for polydisperse coils. The intercept of the curve at the origin is $1/M_w$, that of the asymptote $1/2M_n$. The slope of the curve at the origin $\tan \alpha_0$ is proportional to the z-average (equation 42), that of the asymptote $\tan \alpha_\infty$ to the number average of the square of the gyration radius (equation 45).

to that of the coil with a non-vanishing persistence length. The precision of the measurements is usually not sufficient for discriminating both cases, although the P^{-1} curve of coils with a large persistence length may come closer to the curve for rods than that of polydisperse coils.

The slope of the asymptote α_∞ gives the number average gyration radius

$$\rho_n^2 = M_n \lambda_0^2 \tan \alpha_\infty / 8\pi^2 n^2 \qquad \ldots.(48)$$

The intercept and the slope of the asymptote may be interpreted as number averages of the mol. wt. and gyration radius only in the case of random coils. BENOIT *et al.* (1954) have investigated an unusually polydisperse unfractionated sample of cellulose nitrate in acetone which they have chosen for the reason that the molecule is certainly unbranched and the persistence length can still be neglected, so that all the curvature of the P^{-1} curve is due to polydispersity. They found $M_w = 273{,}000$, $R_z = 2250$ Å and $M_n = 94{,}000$, $R_n = 675$ Å with $b_n = R_n(M_0/M_n)^{1/2} = 38$ Å in perfect agreement with the $b_n$value of the fractionated samples.

For *rods* with constant basis the intercept of the asymptote in the $cK\varphi/R$ plot against $\sin \theta/2$ yields $2/\pi^2 M_n$, i.e. the number average mol. wt.

$$M_n = \pi^2/(2 \times \text{intercept of the asymptote}) \qquad \ldots.(49)$$

and the slope $4n/\lambda_0(M/l)_w$, i.e. the weight average mass density of the rod (CASASSA, 1955). The length hence becomes

$$l_w = M_w \lambda_0 \tan \alpha_\infty / 4n \qquad \ldots.(50)$$

A review of particle scattering factors in polydisperse systems can be found in the articles of GOLDSTEIN (1953), RICE (1955) and LOUCHEUX *et al.* (1958).

Branching not only decreases the ratio between R and ρ of the linear macromolecule (equation 41) but even makes the meaning of the end-to-end distance very uncertain and unimportant as soon as there are either many very long branches or such a tree-like structure that it is hard to define the main chain representing the back-bone of the molecule. As a measure of branching very often the square of the ratio of the gyration radii of the branched molecule (ρ^2) to that of the unbranched (ρ_0^2) of the same mol. wt. is introduced

$$g = \rho^2/\rho_0^2 \qquad \ldots.(51)$$

Although there is obviously no change in the connection of ρ^2 and the initial slope of P, the angular dependence is different from that of the unbranched molecule. For the special case of a cruciform molecule with Gaussian chain element distribution BENOIT (1953) has calculated the angular function P^{-1} which has a marked upward curvature and is situated higher than that for the unbranched coil (Fig. 11). Other shapes can also be calculated but the results are not very helpful because the actual samples of branched polymers are as a rule extremely

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polydisperse, the ratio between the number and weight average mol. wt. amounting often to 1:10 or still higher, and the polydispersity being also not efficiently reducible by fractionation. The superimposed influence of branching and polydispersity on the scattering cannot be separated. From the initial and asymptotic slope of the $cK\psi/R$ curve the ratio of the z- and weight-average mol. wt. multiplied by the $(z+1)$-average of the branching ratio is obtained (MOORE, 1956)

$$\frac{\tan \alpha_0}{\tan \alpha_\infty} = \frac{2}{3}\frac{M_z}{M_w} g_{z+1} = \frac{2}{3}\frac{M_z}{M_w}\left(\frac{\rho^2}{\rho_0{}^2}\right)_{z+1} \qquad \ldots.(52)$$

The branching and the polydispersity hence partly compensate each other. The resultant distortion of the angular dependence P also

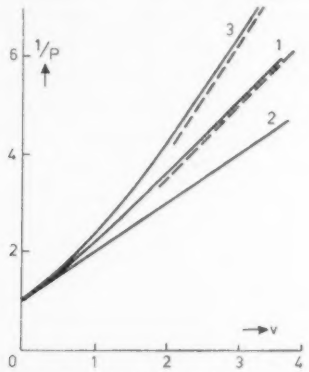

Fig. 11. P^{-1} against v (equations 37 and 40) for random coils (1), polydisperse coils (2) according to equation 46 (Fig. 9), and cruciform molecules (3) (from BENOIT, 1953).

prevents the use of the dissymmetry or dispersion method for the determination of the gyration radius as long as the generally unknown relationship between the initial slope and z or q or Q for the system under investigation is not determined by measuring P in the whole angular range.

The combination of light scattering with intrinsic viscosity makes a good method for studying the branching of polymers. Polyethylene with long side chains was investigated in that way by BILLMEYER (1953), MOORE (1956) and TREMENTOZZI (1957). They found in the fractions extremely broad polydispersity which was in some cases even broader than in the initial unfractionated material. As a further example the investigation of branched polystyrene by JONES *et al.* (1956) has to be mentioned.

Optical anisotropy of the particle (anisotropy of the material: *intrinsic anisotropy* and anisotropy due to the shape of the particle: *shape anisotropy*) influences the angular dependence and the depolarization of the scattered light. As long as the particles are much smaller

than the wave-length in the medium ($< \lambda_0/10n$), the finite depolarization ratio observed with unpolarized primary beam at 90° or with vertically polarized primary beam at any angle, is a sure indication of the optical anisotropy of the particle. With increasing dimensions that is no longer true and one has to extrapolate to $\theta = 0$ to obtain the net influence of the optical anisotropy of the particle without any interference by the depolarization due to the particle dimensions.

The effect was studied by HORN *et al.* (1951) for large rod-like particles. They have shown that the scattering intensity decreases faster (slower) with the angle than for isotropic material when the polarizability in the direction of the rod axis is smaller (larger) than in the perpendicular direction. The measurements on tobacco mosaic virus by HORN and BENOIT (1953) interpreted in terms of this theory yield 2300 ± 200 Å for the length of the virus, in good agreement with the electron microscope value. The relative difference in polarizability $\Delta\alpha_r = (\alpha_1 - \alpha_2)/(\alpha_1 + \alpha_2) = (\delta^2/2)^{1/2} = +0{\cdot}3$ was determined from the extrapolated depolarization ratio $\Delta_v = 0{\cdot}0545$ at $\theta = 0$ which, independently of particle size, is given by equation (16). The positive sign was inferred from the measurement of the electrical birefringence.

In the case of randomly coiled linear macromolecules the depolarization is rather low, much lower than that of the monomer and decreases with increasing mol. wt., but approaches a finite limit at infinite degree of polymerization, as was demonstrated by the investigations of LOTMAR (1938) on methylcellulose and nitrocellulose, DOTY and KAUFMAN (1945) on polystyrene, polyvinylchloride and cellulose acetate, FRISMAN and ZVETKOV (1946) on polystyrene during polymerization and DOTY and STEIN (1948) on polystyrene, polyvinylchloride and polyvinylalcohol in good and poor solvents. In good solvents Δ_v is larger than in poor solvents and the opposite is true for Δ_h. For cellulose acetate in acetone Δ_u is 0·045, 0·028 and 0·019 at $M = 65{,}000$, 90,000 and 158,000 respectively. Polystyrene ($M = 500{,}000$) has $\Delta_u = 0{\cdot}055$ in toluene and 0·007 in butanone. From his model of the Gaussian coil KUHN (1946) deduced the following expression for the optical anisotropy

$$\delta_M^2 = \delta_S^2(sM_0/M) \,.\, z^2/2(z - 1 + e^{-z}) \qquad \ldots.(53)$$

where $z = 4\pi^2 n^2 R^2/3\lambda_0^2$

with the asymptotic value $\delta_\infty^2 = \delta_s^2 \,.\, 2\pi^2 n^2 A_m^2/3\lambda_0^2$

δ_s^2 = optical anisotropy of Kuhn's statistical chain element

s = number of monomers in the element

A_m = length of the element

M_0 = molecular weight of the monomer.

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The actual behaviour only partly agrees with the theoretical predictions.

When the number of segments is small and still more in the case when the molecule is smaller than the segment with s monomers, the depolarization is intimately connected with the mutual orientation of the monomers governed by the valency angle and the rotation restrictions around the valency bond. In this case of very low mol. wt. the investigation of the depolarization could give additional information on the structure of the chain and probably furnish a new method for the determination of mol. wt. (see for instance WEILL, 1958).

2. *Multicomponent systems*

2.1. *Binary solvent.* When the solvent is a mixture of two or more liquids—the most important case is the mixture of a solvent with a precipitant—the concentration of them may be different in the bulk of the solvent and on the surface of the particle where the molecules of the better solvent will accumulate. As a consequence of this selective adsorption the index of refraction n' near the particle differs from that in the undisturbed solvent (n). According to EWART *et al.* (1946) the factor $\mathrm{d}n/\mathrm{d}c$ in K and H has to be replaced by

$$\mathrm{d}n'/\mathrm{d}c = \partial n/\partial c + \alpha \partial n/\partial \phi \qquad \ldots.(54)$$

where $\alpha = -\partial\phi'/\partial c$ with ϕ' and ϕ the partial volumes of the better adsorbed component of the solvent on the surface of the particle and in the undisturbed parts of the solvent respectively. The factor $\partial n/\partial\phi$ means the change of the refractive index of the solvent with the change of the volume fraction of the better solvent. In a solvent-precipitant mixture α is always positive.

With this correction, the extrapolated reduced intensity reads

$$R(0)/c = (4\pi^2 n^2 M/N\lambda_0^4)(\partial n/\partial c + \alpha \partial n/\partial\phi)^2 \psi(0) \qquad \ldots.(55)$$

When both components have the same index of refraction then $\partial n/\partial\phi = 0$ and equation (52) becomes identical with equation (9), i.e. the mol. wt. determination according to equation (20) and (21) is not affected by the composition of the solvent. Such a case is very nearly realized in the polystyrene, butanone, *iso*propanol system where $\partial n/\partial\phi = 0{\cdot}0004$ is negligibly small as compared with $\partial n/\partial c = 0{\cdot}219$. In the system polystyrene, benzene, methanol, however, $\partial n/\partial c = 0{\cdot}108 + 0{\cdot}1447(1 - \phi)$ and $\partial n/\partial\phi = 0{\cdot}16$. In order to explain the experimental data α had to be assumed to be of the order of unity, slightly increasing with methanol concentration. In this case the addition of the precipitant increases the intensity of the scattering which, however, is not caused by the association effect but is a purely optical phenomenon. It is characterized by the fact that it persists at infinite dilution, whereas the association decreases with dilution and with rise of temperature. But the scattering can also be reduced when $\partial n/\partial c$

and $\partial n/\partial \phi$ are of opposite sign as in the case of the system polystyrene, butanone, 1:3-dichloro*iso*propanol with the respective values 0·165 and − 0·102.

The selective adsorption of the solvent component in precipitant mixtures, introducing the correction factor of equation (52), makes less easy the interpretation of light scattering data in terms of mol. wt. and particle dimensions on addition of the precipitant. A shift in the ordinate intercept is mainly an optical phenomenon and only part of it can be attributed to the association effect which still remains after the contribution of selective adsorption has been properly taken into consideration.

2.2. *Polyelectrolytes.* In a solution of colloidal cations of mol. wt. M, charge p and concentration c (g/ml) in the presence of y equiv. per ml of univalent anions and x equiv. per ml of univalent cations, i.e. of x mole/ml of added low mol. wt. electrolyte, the low molecular anions being at the same time the gegenions of the polyelectrolyte, the conservation of charge requires

$$y = x + pc/M \qquad \ldots.(56)$$

From the general theory of fluctuations in multicomponent systems (Zernike, 1918; Brinkman and Hermans, 1949; Kirkwood and Goldberg, 1950; Stockmayer, 1950), Edsall *et al.* (1950) and Prins and Hermans (1955) deduce for the turbidity of a dilute polyelectrolyte solution*

$$\frac{HcQ}{\tau} = \frac{1}{M}\left(1 + \frac{p^2c/M}{2x + pc/M}\right) \qquad \ldots.(57)$$

under the mostly valid assumption that the only significant contribution to scattering is due to the colloid component and that the ionic strength as measured by y is not too low. It was shown by Edsall *et al.* (1950) that it holds good for the protein solutions studied by them. The main feature of this result is the charge dependent term which rapidly decreases with increasing dilution and mol. wt. of the particle but also with decreasing ionization of the polyelectrolyte and with added low mol. wt. salts. Hence the behaviour of polyelectrolytes in solutions of high content of low mol. wt. electrolyte is much the same as for uncharged particles. In pure water and in dilute salt solutions, however, the influence of the charges is appreciable and strongly reduces the scattering intensity. In that case, however, equation (57) has to be modified by a further term measuring the long range coupling of the density fluctuations due to the charged particles. (Timasheff *et al.*, 1955, 1957.)

* This result agrees with the case D treated by Mysels (1954). See also Doscher and Mysels (1951), Hutchinson (1954), Hutchinson and Melrose (1954) and Mysels (1955).

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In the case of linear polyions the charges in the ionized state are so high and hence, in the absence of an ionic atmosphere so much reduce the scattering and change its angle dependence, that it becomes practically impossible to measure and interpret the observations with any degree of precision.

The angular dependence P yields information on the size and shape of the charged particle. Because the dimensions of the coiled macromolecules are strongly dependent on the charge of the particle (ionization degree) and on the ionic strength of the solvent the measurement at different concentrations have to be made at constant charge on the polyelectrolyte and at constant concentration of low molecular weight gegenions (y). For that purpose at dilution low mol. wt. electrolyte has to be added in such amounts that y remains unchanged (*isoionic dilution*: PALS and HERMANS, 1948).

Detergent solutions are an important special case of polyelectrolyte solutions. Micelle formation starts at the critical concentration c_0 of the ionic detergent. Extensive studies of the equilibrium between monomers and micelles have shown that beyond c_0 the monomer concentration remains practically constant on further addition of the soap. Hence $c - c_0$ is the concentration of the micelle which can be treated as independent charged particles (polyelectrolyte). DEBYE (1949) has shown that with this assumption the mol. wt. of the micelles can be deduced in the usual way from light scattering measurements. According to PRINS and HERMANS (1956), at a sufficiently low micelle concentration and at a not excessively high concentration of the extraneous salt,

$$(c - c_0)H/\tau = g/M$$

where
$$g = (c_0/M_1 + x)/[(1 - \alpha)c_0/M_1 + (1 - f\alpha)x] \quad \ldots.(58)$$

with M_1 = mol. wt. of the detergent monomer, x the number of equiv. per ml of the extraneous ions of the same sign as the detergent monomers, α the ionization degree of the micelle and f the ratio between the refractive index increments at the addition of a mole of the salt and the monomer respectively.

3. *Concentration dependence of light scattering*

The scattering of light is due to the fluctuation of optical density in the sample. From the correlation between the statistical fluctuation in concentration and the fluctuation of polarizability EINSTEIN (1910) derived the excess turbidity of the solution as function of the change of the osmotic pressure Π with concentration

$$\tau = \frac{32\pi^3 n^2}{3N\lambda_0^4} \frac{(c\ \mathrm{d}n/\mathrm{d}c)^2}{(c/RT)\ \mathrm{d}\Pi/\mathrm{d}c} \quad \ldots.(59)$$

which is valid as long as the scattering by the single particle is isotropic. For larger particles, equation (59) only holds for the reduced scattering intensity at $\theta = 0°$. From*

$$\Pi/RT = c/M + Bc^2 + \ldots \qquad \ldots.(60)$$

one obtains

$$cH/\tau = 1/M + 2Bc + \ldots \qquad \ldots.(61)$$

The slope of the cH/τ plot against c is twice the second virial coefficient B and the reciprocal of the ordinate intercept the weight average mol. wt. A large B, which makes the extrapolation to $c = 0$ less easy, can be avoided by a precipitant solvent which in turn complicates the

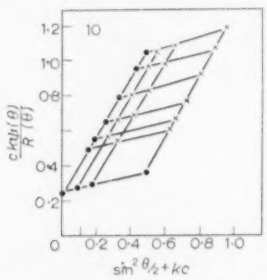

Fig. 12. A typical Zimm plot of polymethacrylic acid, $M = 4{\cdot}1 \times 10^6$ for the 6 per cent ionized form in 0·16 M NaCl solution.

interpretation by the selective adsorption of the good solvent on the surface of the particle. The "ideal" solvent with $B = 0$ is a very poor solvent, the dissolved particles being at the threshold of precipitation.

At finite concentration, the interaction of the optical fields of the particles in the solution markedly influences the intensity and polarization of the scattered light. Therefore one has first to extrapolate all the measurements to infinite dilution before any comparison with the theory of the single particle scattering and any determination of the particle properties can be performed.

The extrapolation of the angular dependence of R to zero concentration is mostly done by the Zimm plot (Zimm, 1948a) in which the measured $cK\psi(\theta)/R(\theta)$ is plotted against $kc + \sin^2(\theta/2)$ (Fig. 12). One extrapolates at constant θ to $c = 0$ and at constant c to $\theta = 0$, the common intercept of both extrapolated curves on the ordinate yields $1/M_w$. The slope of the curve $\theta = 0$ divided by k gives $2B$; the slope of the curve $c = 0$ multiplied by $3\lambda_0^2/16\pi^2n^2$ the square of the gyration radius.

* From statistical mechanical treatment of dilute polymer solutions Fox *et al.*, (1951) deduce the third term in equations (57) and (58) as $5B_2^3M/8$ and $15\ B_2^3M/8$ respectively and prove this relationship in *cyclo*hexane solutions of several poly*iso*butylene fractions.

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The Zimm plot is based on Zimm's single contact approximation of reciprocal scattering intensity

$$cK\psi(\theta)/R(\theta) = 1/MP(\theta) + 2Bc + \quad \ldots.(62)$$

according to which all the extrapolation curves at constant θ ought to be parallel. This is evidently not so in a great many cases. By treating the intermolecular contribution according to the smoothed Gaussian sphere model for the single macromolecule in solution ALBRECHT (1957) and FLORY and BUECHE (1958) obtain instead of equation (62) the expression

$$cK\psi(\theta)/R(\theta) = 1/MP(\theta) + 2b(\theta)Bc + \quad \ldots.(63)$$

with the angle dependent factor $b(\theta)$ falling appreciably below unity at higher angles for large molecules.

With particles having very high mol. wt., e.g. desoxyribonucleic acid, the double extrapolation of the Zimm plot is frequently difficult and uncertain. In such a case YANG (1957) proposes a plot of $cK\psi(\theta)/R(\theta)\sin^2(\theta/2)$ over $1/\sin^2(\theta/2)$ yielding a series of curves having c as parameter and a common intercept yielding the number average gyration radius. The common intercept of the asymptotes yields its z-average. The initial and asymptotic slope of the extrapolated $c = 0$ curve gives M_n and M_w. YANG applies this method to a cellulose nitrate measured by BENOIT *et al.* (1954) and finds with $M_n =$ 220,000 and $M_w = 630{,}000$ a very good agreement with their published results of 211,000 and 640,000.

When the dissymmetry or dispersion only is observed, the extrapolation to zero concentration yields the intrinsic dissymmetry coefficient z and the intrinsic dispersion coefficient q respectively. As long as one is only interested in the gyration radius one is not obliged to know the absolute values of the concentration of the samples. A stock solution of indefinite concentration can be taken and measured at dilutions of unknown extent. If the observed data of z are plotted against the excess scattering at 90° the intercept on the ordinate gives the extrapolated value for zero concentration (CLEVERDON *et al.*, 1955).

Polyelectrolyte solutions may show a rather complicated concentration dependence (see, for instance, KIRKWOOD and TIMASHEFF, 1956). At low ionic strength, e.g. in salt-free solutions, a plot of $Kc\psi/R$ against $c^{1/2}$ yields very nearly straight lines (TIMASHEFF *et al.*, 1957; KIRKWOOD and SHUMAKER, 1952). The large interaction of highly charged particles (preventing independent density fluctuation and hence reducing scattering intensity) increases the order in the solution and gives rise to strange effects as, for instance, the decrease of the dissymmetry to less than 1, the appearance of minima in P, and so on.

As already mentioned the dilution has to be made in such a way

that neither the charge (dissociation) of the particle nor the ionic strength of the solution are changed because both may influence the shape of the particle. This precaution is especially necessary in solutions of linear macromolecules which uncoil at high ionization and contract at high ionic strength of the solution.

IV. Experimental Results

1. *Large particles*

A great many linear and globular polymers have been investigated by the light scattering technique. Monodisperse sols (sulphur, polystyrene latex), polystyrene and polymethylmethacrylate solutions were preferred as model substances for investigating the feasibility of the method, for the determination of shape and size in different solvents and variation with the temperature, for the study of polydispersity and so on. Some samples of polystyrene distributed by Prof. Mark were independently measured in different laboratories all over the world and thus the reliability of the method checked by comparison of the results obtained (Frank and Mark, 1953, 1955). The agreement was very satisfactory and encouraging as far as both the mol. wt. and the gyration radius were concerned.

Spherical particles, with non-negligible difference in refractive index, were investigated among others by Dandliker (1950, polystyrene latex), Atherton and Peters (1953b, polycellosolve methacrylate and polyvinylchloride latex); Chin *et al.* (1955); Heller (1955), Heller *et al.* (1956, 1957 polystyrene latex) and vanadium pentoxide sols by Kerker *et al.* (1954).*

The most important result in the investigation of linear polymers is the direct proof of the dependence of the coil diameter on the solvent and temperature as was suggested by intrinsic viscosity data. The better the solvent the greater R and vice versa. In many cases (Cantow and Bodman, 1955) the coil diameter is a linear function of the second virial coefficient B. In good (poor) solvents R decreases (increases) with increasing temperature. According to Flory (1949) the diameter of the macromolecule at the precipitation equilibrium should be independent of the solvent. The charges on the polyelectrolyte molecule expand the coil increasingly the less they are screened by the ionic atmosphere of the solution (Hermans and Overbeck, 1948a b; Kuhn *et al.*, 1948a b; Hermans, 1949; Künzle, 1949; Katchalsky *et al.*, 1950; Arnold and Overbeck, 1950; Flory, 1953; Katchalsky and Lifson, 1953; Harris and Rice, 1954; Krieger, 1957; Peterlin, 1958).

Shultz *et al* (1956) investigated the crosslinking and degradation

* See also the chapter 1.2 (Mie-scattering).

of polystyrene (PS) and polymethacrylates (PMA) under the influence of irradiation by electrons of 1000 keV. In PS the crosslinking prevails over the chain scission yielding at 9 megareps an increase of M_w from $1{\cdot}15 \times 10^6$ to 26×10^6. In polymethyl methacrylate, however, there is practically no crosslinking so that after a dosage of 11 megareps the initial $M_w = 3{\cdot}20 \times 10^6$ dropped to $0{\cdot}131 \times 10^6$.

The influence of *different solvents* on the coil diameter in PS was studied by DOTY *et al.* (1946); ZIMM (1948c); FRISMAN and KISELEVA (1950); KUNST (1950); DEBYE and CASHIN (1951); TREMENTOZZI *et al.*(1952; anomalous association in non-polar solvents, very likely due to impurities with OH-groups in the chain introduced during polymerization); TREMENTOZZI and BUCHDAHL (1954); TRAP and HERMANS (1954b); OTH J. and DESREUX (1954b); FENDLER *et al.* (1954a b); MIZUTANI (1956); SOBOLEVA *et al.* (1956) and PEAKER (1956: isotactic PS); JONES *et al.* (1956: branched PS). When lowering the temperature in precipitant-solvent mixtures the coil diameter decreases under the values found formerly at the precipitation point (FENDLER *et al.*, 1954a). For a fraction with $M = 1{,}070{,}000$ in toluene–butanone mixture (51:49 weight ratio) the diameter is 300 Å at 52°C as compared with 1090 Å in toluene at 20°C. The value 300 Å just corresponds to a coil with absolutely free rotation around the valency bond. Moreover at the precipitation equilibrium the radius turns out to be dependent on the solvent in contradiction to FLORY's theoretical predictions. NOTLEY and DEBYE (1955) suggest the relation $R^2 = k(M - b)$ with $b = 240{,}000$ for high mol. wt. polystyrene in toluene at 20°. FRANK (1955) and FENDLER and STUART (1956) have investigated light scattering at extreme dilution of PS in order to detect an eventual additional extension of the coil over that found by extrapolation of measurements at higher concentrations. There was no such effect so that the anomalies in η_{sp}/c have to be attributed to adsorption effects on the walls and not to an anomalous extension of the coils at extreme dilution.

Polymethyl methacrylate in different solvents was investigated among others by FRISMAN and KISELEVA (1950) and BISCHOFF and DESREUX (1952, 1953); in butanone in a wide range of mol. wt. from 34,000 to 9,800,000, by BILLMEYER and DE THAN (1955); in benzene, chloroform and acetone with M_w ranging from 28,000 to 7,800,000 by SCHULZ *et al.* (1953), CANTOW and SCHULZ (1954b), CANTOW and BODMANN (1955) and further by BHATNAGAR and BISWAS (1954); in acetone at different temperatures by FENDLER *et al.* (1954); in precipitant solvents by CHINAI *et al.* (1955) and CHINAI and BONDURANT (1956). When the correction due to the non-Gaussian character of the chain (PETERLIN, 1955b) is applied to the R-values of CANTOW *et al.*, a perfect agreement is obtained with the diameters derived from the combination

of sedimentation constant and intrinsic viscosity (PETERLIN, 1955c, 1956). Polyethyl methacrylate was studied by CHINAI and SAMUELS (1956) poly-*n*-butyl methacrylate by CHINAI and GUZZI (1956) and poly-*n*-hexyl methacrylate by CHINAI (1957).

Polyethylene was investigated by BILLMEYER (1953), NICOLAS (1955), MOORE (1956), DUCH and KÜCHLER (1956), KOBAYASHI *et al.* (1957), MUUS and BILLMEYER (1957). The rather polydisperse samples contained more or less branched molecules and frequently also an insoluble component which could not be removed by ordinary means and which was obviously responsible for the irregularity of the results, i.e. too high mol. wt. and size, irregular Zimm plot in unclarified solutions, etc. The width of mol. wt. distribution can be estimated from ratios of weight-to-number average mol. wt. in the range 11–18 (MUUS and BILLMEYER, 1957). DOTY *et al.* (1947) find a very marked trend to association in *polyvinylchloride* solutions in dioxan and *cyclo*hexanone. DOTY *et al.* and LAKER (1957) report a monomolecular solution in tetrahydrofuran. PETERLIN (1955a) studied the influence of branching in terahydrofuran solutions. *Polyvinylbromide* in *cyclo*hexanone and tetrahydrofuran shows no association effects at all (CIFFERI *et al.*, 1957, 1958). *Polyvinylalcohol* was measured by TIMASHEFF *et al.* (1949b), BOSWORTH *et al.* (1952), SHULTZ (1954), CHINAI *et al.* (1955) and VARADAIAH (1956).

Cellulose trinitrate in acetone was studied by BADGER and BLAKER (1949), in the wide range of M_w from 77,000 to 2,640,000; by HOLTZER *et al.* (1954) and in ethyl acetate with $M_w = 41{,}000$–$573{,}000$ by NEWMAN and FLORY (1953) and HUNT *et al.* (1956). The coil is much more extended than in other polymers, the persistence length being 129 Å in acetone and 117 Å in ethyl acetate corresponding to the length of 25 and 23 glucose units respectively. Cellulose acetate in acetone (DOTY and KAUFMAN, 1945; STEIN and DOTY, 1946) with M_w from 52,000 to 163,000 and ethyl hydroxyethyl cellulose in water (MANLEY, 1956) with $M_w = 160{,}000$–$570{,}000$ are still more extended than cellulose nitrate. Cellulose degradation and the formation of a polymer of 5-hydroxy methyl furfural in concentrated sulphuric and phosphoric acid has been investigated by CHOUDHURY and FRANK (1956) and CHOUDHURY (1957).

Light scattering by GR-S solution of very small refractive index increment presents a special anomaly, the amount of scattering being approximately 10 times larger than would be expected (RIVEST and RINFRET, 1950). The reason is the inhomogeneous composition of the polymer (styrene and butadiene monomers). The refractive index of the polymer is the average over all constituents. When it is equal to that of the solvent, the refractive index increment dn/dc will be zero, but in single parts of the molecule the difference in polarizability between the

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solute and the solvent will not vanish having varying positive and negative values according to the local composition of the chain. If the GR-S is homogeneous in composition this effect will be zero and so will the scattered intensity. At any inhomogeneity, however, the scattering will be greater than that calculated from the observed value of dn/dc yielding therefore an apparent mol. wt. which is too large. Hence this effect could be used for studying the composition heterogeneity of GR-S (TREMBLAY *et al.*, 1952). On the other side GR-S samples obtained by emulsion polymerization also contain microgel particles of highly crosslinked material having a mol. wt. of nearly 20×10^6 and a diameter of 2000 Å which cannot be easily removed from the solution and act as very strong light scatterers (BAKER, 1949; MEDALIA, 1951, MEDALIA and KOLTHOFF, 1951).

Polyisobutylene in benzene has a diameter of 1150 Å at $M =$ 1,600,000 (FRISMAN and KISELEVA, 1950), crêpe-rubber in *cyclo*hexene, one of 750 Å at $M_w = 1{\cdot}3 \times 10^6$ (SCHULZ *et al.*, 1956). In Alfin *polybutadiene* with M_w from 5 to 20×10^6 CLELAND (1958) finds a decrease in branching with increasing conversion at polymerization. The molecules of poly-1:1-dihydroperfluorobutyl acrylate (RATHMANN and BOVEY, 1955) with $M_w = 2\text{–}38 \times 10^6$ exhibit increased long chain branching with higher conversion. As a consequence the gyration radius in benzotrifluoride only increases from 335 Å to 1225 Å but M_w goes from 2 to 38×10^6. In a better solvent, methyl perfluorobutyrate, the dimensions increase by nearly 10 per cent as compared with those in benzotrifluoride. Polyvinylpyrrolidone in water was measured by HENGSTENBERG and SCHUCH (1952).

The highly branched *dextran* (RIDDICK *et al.*, 1954; SEDLAČEK, 1954; MASTRANGELO *et al.*, 1955) is much less extended than an unbranched molecule of the same mol. wt. The gyration radius increases more slowly than $M^{1/2}$ in the range of M_w between 400,000 and 2,700,000 (SENTI *et al.*, 1955). Because the mol. wt. determination by light scattering is not influenced by rather large methanol admixture (up to 16 per cent by volume), it permits an accurate characterization of the sample also in the case when the viscosity is already reduced by the presence of methanol (GRAHAM *et al.*, 1956). *Carrageenin* with $M_w = 1{\cdot}7 \times 10^6$ has a nearly rod-like shape with a length of 3700 Å (GORING, 1953). In solvents of varying *p*H *amylose* molecules seem to form stable aggregates (PASCHALL and FOSTER, 1952a b) which are much more highly resistant to precipitation retrogradation the more perfect the helical crystalline form of the amylose preparation (FOSTER and STERMAN, 1956). Potato amylopectin (WITNAUER *et al.*, 1952, 1955) has a very high mol. wt. from 14 to 73×10^6, a very broad spectrum and, due to the large extent of branching, rather low values of the gyration radius. So, for instance, at $M_w = 14$ and 36×10^6 the

gyration radius of 1090 and 1880 Å respectively, is nearly independent of temperature and hydroxyl ion concentration. But the molecules aggregate on addition of 10^{-3} N KCl. Corn amylopectin and its limiting dextrin (Stacy and Foster, 1956; Stacy *et al.*, 1956) behave in substantially the same way, the gyration radius being 2090 Å at $M = 80 \times 10^6$ (amylopectin) and 1340 Å at 29×10^6 (dextrin).

As early as 1935 Putzeys and Brosteaux were able to prove in dilute solutions of *globular proteins* with M between 34,000 (ovalbumin) and 5,000,000 (haemocyanin) that the intensity of the scattered light at constant concentration is proportional to the mol. wt. of the solute. Among the first determinations of mol. wt. of proteins are those of globular glycogenes by Schulz (1944) and of ovalbumin by Heller (1945) and Heller and Klevens (1945).

There have been many investigations of human serum *albumin* (Edsall *et al.*, 1950; Halwer *et al.*, 1951; Edsall and Dandliker, 1951) which is very likely a rod-like particle with the length of 540 Å; of bovine serum albumin (Doty and Steiner, 1949, 1950; Dandliker, 1954; Geiduschek, 1954; Timasheff *et al.*, 1957); of human and bovine mercaptalbumin (Timasheff *et al.*, 1957); of beta-globulin (Koechlin, 1952) and of gamma-globulin (Schönenberger, 1955; Heide, 1956). Special attention was given to the charge-effect in salt free solutions (isoionic particles, i.e. particles with their own gegenions only). Although the single serum albumin molecules carry a net charge of nearly 50 protons, the determination of mol. wt. by light scattering is influenced by less than 1 per cent. Conalbumin, with a nearly vanishing net charge (Timasheff and Tinoco, 1957) turned out to be a truly monodisperse protein with a mol. wt. of *c.* 76,500. Fuller and Briggs (1956), however, find $M = 82{,}400$. Bier and Nord (1949a) studied aggregation phenomena in egg albumin, Heilweil and van Winkle (1955) its interaction with polyvinyl butylpyridinum bromide. In combining light scattering measurements on the *myosin* molecule with sedimentation, viscosity and streaming birefringence Holtzer (1956) and Holtzer and Lowey (1956) conclude that the appropriate model is a rod 1650 Å long, 26 Å thick and of mol. wt. 530,000 in rather good agreement with the older measurements of Mommaerts (1951) yielding $M = 850{,}000$ and $L = 1500$ Å. In a long series of studies on *fibrinogen* and *fibrin*, Casassa (1955, 1956a b). Casassa and Billick (1957) were able to show that the molecule of *fibrinogen* (monomer) is very likely a thin rod-like particle of mol. wt. 340,000 and length 520 Å with a tendency to lateral dimerization at increased concentration. Under the enzymatic action of thrombin it polymerizes into cylindrical rods of indeterminate length, but at least of the order of 5000 Å, and mass to length ratio 2·3 times that for monomeric fibrinogen. Steiner and Laki (1951a b) and Laki and

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STEINER (1952) have found $M = 540–560 \times 10^3$ and $L = 840–850$ Å, HOCKING *et al.* (1952) $M = 407{,}000$ and $L = 670$ Å (ellipsoids). *Elinin*, a lipoprotein from human erythrocytes, has $M = 40$ million and $L = 3000$ Å (DANDLIKER *et al.*, 1950). DOTY *et al.* (1956) investigated *poly-γ-benzyl-L-glutamates* in various solvents in which they are soluble without association. In the first group of solvents (dimethylformamide, cresol, chloroform–formamide) and for M over 20,000 the length is proportional to the mol. wt. with the length per residue being 1·5 Å. That means that the configuration is that of the α-helix of PAULING and COREY with the diameter 14·9 Å. The helix is rather rigid and rod-like up to $M = 300{,}000$ and exhibits a very small amount of flexibility at $M = 800{,}000$. In the extremely solvating dichloroacetic acid, however, the shape of the molecule is a random coil. Moderately high mol. wt. *gelatine* ($M = 96{,}000$) has been found to be molecularly dispersed in 0·15 N NaCl at 40°C and in 2 N KCNS solution (BOEDTKER and DOTY, 1954), the molecules being random coils of 420 Å diameter. When the temperature is lowered aggregate formation occurs with aggregate weights of several millions instead of gelation when the gelatine concentration is below a critical value. VEIS and COHEN (1957), however, determine for two commercial gelatins of $M_w = 330{,}000$ a gyration radius of 110 Å in isoionic and of 450 Å in 0·2 M KCl solution. For *collagen* the building unit turns out to be a rod-shaped particle of 3000 Å length and 14 Å diameter having a mol. wt. of 340,000 (BOEDTKER and DOTY, 1955). Sodium chondriotin sulphate purified from bovine hyaline cartilage and treated with proteolytic enzymes to remove trace impurities has a light scattering mol. wt. of 50,000 and appears to be very nearly monodisperse (MATHEWS, 1956).

Desoxyribonucleic acid was very often investigated by light scattering (SIGNER and SCHWANDER, 1949; SMITH and SHEFFER, 1950; RILEY and OSTER, 1950, 1951; BUNCE, 1951; DOTY and BUNCE, 1952; REICHMANN *et al.*, 1952, 1953, 1954; KATZ, 1952; STEINER, 1952; CONWAY and BUTLER, 1952; GEIDUSCHEK and DOTY, 1952; ROWEN, 1953; ZAMENHOF *et al.*, 1953; THOMAS, 1954, 1956; DOTY and RICE, 1955; BROWN *et al.*, 1955; ALEXANDER and STACEY, 1955b; SADRON, 1955; REICHMANN, 1956; THOMAS and DOTY, 1956; CAVALIERI *et al.*, 1956; GEIDUSCHEK, 1956; POUYET *et al.*, 1956; BUTLER *et al.*, 1957; ROSOFF *et al.*, 1957; HERMANS, 1958). The molecule, having a mol. wt. between 5·8 and 7·7 $\times 10^6$ (older preparations from 3·5 to 6·8), can be represented by a rather stiff double helix which due to its great extended length between 18,000 and 24,000 Å is nearly randomly coiled with a diameter of nearly 5500 Å (at *p*H 6·5), the persistence length being about 280 Å (Fig. 13). In methanol the diameter is only one third of that in water (GEIDUSCHEK, 1956). Nearly one-third of the samples, however, yield

a mol. wt. twice as high ($11{\cdot}6$–$16{\cdot}5 \times 10^6$). After irreversible denaturation at $p\mathrm{H} < 3{\cdot}1$ or $> 11{\cdot}8$ or at higher temperature the diameter only decreases to nearly 2400 Å, the mol. wts. remaining unchanged. Denaturated samples show a polydispersity in shape besides the usual polydispersity in mol. wt., which prevents any more detailed interpretation of the scattering data. One assumes that at denaturation the regular double helix structure is damaged in single points or in

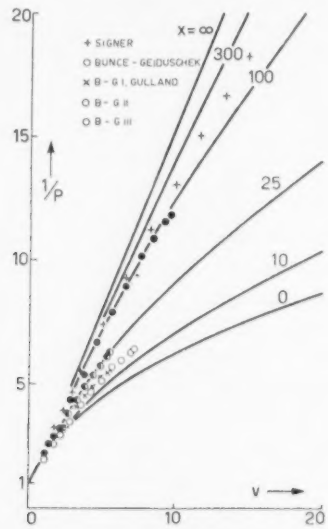

Fig. 13. P^{-1} for different preparations of DNA against v (from PETERLIN, 1953c). The experimental points fit the theoretical curves for coils with finite persistence length rather well. A comparison with Fig. 5, however, shows that polydispersity also yields similar P^{-1} curves. The precision of the experimental data is not sufficient for a convincing decision as to that which is due to polydispersity and that which is due to persistence length. The measurements were made by BUNCE (1951).

longer sections where the secondary bonds between the side groups are broken. In these areas the molecule becomes practically completely flexible as compared with the very stiff undamaged double helix. In such a way after partial denaturation the single particle of DNA consists of stiff segments of varying length linked together by longer or shorter nearly freely flexible loose parts of the double chain.

Polymethacrylic acid (PMAA) was studied by OTH and DOTY (1952), OTH (1954), TRAP and HERMANS (1954a) and ALEXANDER and STACEY (1955a). With increasing ionization the coil diameter rapidly increases. In a sample with $M_w = 90{,}300$ at ionization degree $\alpha = 0{\cdot}001$ the diameter is about 200 Å, at $\alpha = 0{\cdot}155$ it is 710 Å and at $\alpha = 0{\cdot}71$ it reaches 1180 Å. In a salt free solution the scattering of the ionized form could

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not be obtained at concentrations sufficiently low for extrapolation to infinite dilution. A 60 per cent neutralized sample with $M_w = 1{\cdot}02 \times 10^6$ shows a drop in the coil diameter from 2030 Å to 1900 Å and 1720 Å when the NaCl concentration increases from 0·06 N to 0·08 N and 0·8 N respectively. By going from methanol to 0·05 N HCl water solution the coil diameter of a sample with $M_w = 195{,}000$ changes from 735 Å to 780 Å. TREMENTOZZI (1954) used pure ethanol and ethanol with 0·125 and 0·250 N LiBr. From the small differences in R, he concludes that the molecules of PMAA with M_w between 42,000 and 383,000 are nearly unionized. ARNOLD and CAPLAN (1955a b) mainly confirm older results on chain expansion. The ionized molecule reaches a maximum volume at finite concentration and then decreases with increasing concentration. EHRLICH and DOTY (1954) investigated copolymers of PMAA and 2-dimethylaminoethyl methacrylate carrying charges of both sign. Due to the intramolecular attraction forces the molecules are less extended as purely anionic or cationic polyelectrolytes.

Carboxymethylcellulose with $M_w = 440{,}000$ was studied by SCHNEIDER and DOTY (1954). When the ionic strength decreases from 0·5 to 0·005 equiv. per litre the coil diameter increases from 2370 Å to 3350 Å in good agreement with the theory of HARRIS and RICE (1954). TRAP and HERMANS (1954a) find for a sample with $M_w = 86{,}000$ a similar increase in the diameter from 415 Å to 730 Å, in excellent agreement with intrinsic viscosity data, when the ionic strength decreases from 0·19 to 0·000 75 equiv. per litre. Both series of coil expansion are considerably lower than predicted by the theories of HERMANS and OBERBECK (1958a b) and FLORY (1953), whereas the theories of KUHN *et al.* (1948a b), KÜNZLE (1949), KATCHALSKY *et al.* (1950) and KATCHALSKY and LIFSON (1953) can provide no predictions for this case at all.

On *polyvinylalcoholsulphate*, a strong electrolyte, TERAYAMA (1956) finds at low ionic strength a minimum in the angular distribution of the scattered light (θ between 100° and 110°) due to large intermolecular interaction of long range repulsive forces among polymeric ions. In solutions of high ionic strength such a minimum disappears.

The diameter of the molecule of *gum arabic*, $M_w = 1{\cdot}0 \times 10^6$, increases from 1050 Å in the uncharged state to 2400 Å at maximum charge (VEISS and EGGENBERGER, 1954). *Hyaluronic acid* has a diameter of 2200 Å at M_w $3{\cdot}1 \times 10^6$ and ionic strength 0·12. At decreased ionic strength 0·012, however, the mol. wt. is $4{\cdot}8$–$6{\cdot}5 \times 10^6$ very likely due to aggregation (LAURENT and GERGELY, 1955).

The temperature dependence of light scattering by *hydrogen bonding* polymers, i.e. PMAA, polymethacrylamide, polyacrylic acid, and polyacrylamide was studied by SILBERBERG *et al.* (1957). The mol. wt. does not change, but the second virial coefficient decreases with increasing temperature in the first case and increases in the other three

cases. BERKOWITZ *et al.* (1958) have investigated poly-4-vinylpyridine in ethanol. Polymers and copolymers of vinylbenzyl trimethylammonium chloride with acrylamide and acrylonitril investigated by JONES and GOETZ (1957) do not indicate aggregation although the monomer exhibits micellar behaviour in conductivity measurements.

The first *micellar* mol. wt. determination was made by DEBYE (1949) on solutions of alkyl quaternary ammonium salts, e.g. normal alkyl trimethylammonium bromides, and amine hydrochlorides. From the dissymmetry of the scattered light DEBYE and ANACKER (1951) determined the shape and mol. wt. of the micelles of hexadecyltrimethylammonium bromide. ANACKER (1953) has investigated the double paraffin chain salts, octyltrimethylammonium octanesulphonate and decanesulphonate. PHILLIPS and MYSELS (1955) and KUSHNER and HUBBARD (1955) measured pure sodium dodecyl sulphate in water and in salt solutions and TARTAR and LELONG (1955) measured 13 normal paraffin chain salts with anionic and cationic chains in aqueous solution, alkane sulphonates, alkyl sulphates and alkyl quaternary salts. TRAP and HERMANS (1955) investigated cationic soaps, SUNDARAMAN (1954) sodium oleate in xylene during and after gel formation.

In all cases the critical micelle formation concentration decreases with increasing salt concentration. The micelle size and charge, however, increase approaching more or less rapidly an upper limit, the charge generally increasing less than the number of monomers in the aggregate. For instance, in sodium lauryl sulphate in pure water, the micelle contains 95 monomers and has a charge of 16 units, at 0·002 N NaCl it has 100 monomers, at 0·10 N 117 and at 0·40 N 132 monomers and a charge of 17 units. Very likely the charge also changes with increasing micelle concentration thus making the extrapolation to infinite dilution still less easy. The number of monomers in the micelle varies within rather wide limits from 30 in the pure water solution of sodium octansulphonate to 700 in the case of octyltrimethylammonium decanesulphonate in 0·178 N KCl solution (TARTAR, 1955).

But these results are not yet generally accepted and there remains considerable disagreement concerning the size of the micelle and its dependence on concentration of added electrolytes. A soap of fixed micelle size is the *polysoap*, i.e. a polymer composed of soap-like monomers, e.g. the copolymer of styrene and potassium styryldecanoate, in 1:1 mole ratio, studied by SINHA *et al.* (1957). At mol. wt. between 80,000 and 1,770,000 the charges turn out to be very low, much lower than that of micelles of nearly the same size.

Non-ionic detergents behave in a similar manner to the ionic detergents, but the absence of an electric charge eliminates a complicating factor in evaluating light scattering data. Triton X-100, a polyoxyethylene condensate of an octylphenol, was investigated by KUSHNER

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and HUBBARD (1954) and by KUSHNER *et al.* (1957) in water and in 0·04 and 0·12 N NaCl solutions. Formation of micelles appears to begin at the lowest concentration, although the monomer saturation concentration does not occur until about 0·003 g/ml. With this value for the critical micelle formation concentration, light scattering yields the value 90,000 for the micelle mol. wt., the mol. wt. of Triton X-100 being 650. With higher M of the detergent molecule the mass of the micelle also increases. The presence of neutral electrolyte has no effect on the properties of the micelles.

On two *azo dyes*, orange II and benzopurpurin 4B, FRANK (1957) has observed micellar particles of large size only in the presence of added salt, the degree of association being much greater for benzopurpurin where $M_w = 1{,}900{,}000$ was determined at 25°C and 0·04 M KCl. The maximum mol. wt. for orange II reaches 37,000 at 5°C and 0·4 M KCl. The micelles are rodlike particles. Micelle formation of benzopurpurin takes place already in the absence of salt if the pH approaches the isoelectric point.

2. *Turbidimetric titration*

With addition of increasing amounts of a precipitant to the solution of a non-fractionated polymer sample the components with the highest mol. wt. will coagulate first. The coagulation creates large particles which can be easily detected by light scattering or by turbidity measurement. The amount of the coagulate can be estimated from the turbidity which is proportional to the volume of the coagulate. For larger particles—they are generally assumed to be spherical—the correction factor Q is derived from the observed dissymmetry (OTH and DESREUX, 1954). The determination of the mass of the coagulate, however, requires knowledge of the particle density. Under the assumption that the latter does not change during precipitation it can be derived from the data for completed precipitation. Using precipitation data on narrow fractions the polydispersity of the investigated sample can be obtained (see also CLAESSON, 1955). The method introduced by MOREY and TAMBLYN (1945) in the study of cellulose acetate butyrate, and later applied to nitrocellulose by OTH (1949), to cellulose acetate by BISCHOFF and DESREUX (1951), to polymethylmethacrylate by HARRIS and MILLER (1951), to polyvinylacetate by MOREY *et al.* (1951), to polyvinylchloride by OTH and DESREUX (1954a) and by KRAŠOVEC *et al.* (1957), to graft-polymers by MELVILLE and STEAD (1955) and to polyvinylpyrrolidone by SCHOLTAN (1957a b), gives rather good information on the system investigated although due to the many uncertainties in the assumption, very accurate results cannot be expected. But it can be well used for rapid orientation and for the study of changes in the chemical and mechanical structure due to

different treatments, e.g. irradiation, chemical attack, different polymerization conditions and so on. A good survey of the potentialities was given by HENGSTENBERG (1956), but the method is not simple and requires a very accurately reproducible experimental arrangement, temperature, uniform distribution of the precipitant and a precise calibration by monodisperse fractions.

V. EXPERIMENTAL METHODS

1. *Turbidity measurements*

The most direct method is by determination of incident and transmitted intensity of the solution and the solvent in such an instrument as the Beckman spectrophotometer. Many photometers already measure the difference in absorption between the solution and the solvent. At known length of the cell the excess turbidity is then directly obtained from the instrument reading; but this direct method for turbidity is only applicable when the turbidity is fairly high so that the ratio $I_0(x):I_0(0)$ differs from unity by at least a few per cent so that it can be determined accurately enough.

It is often difficult to prove that all the change in transmitted intensity is due to scattering and none to true absorption or fluorescence. Measurement at different wave-lengths is almost essential in this connection. If the attenuation varies as the inverse fourth power of the wave-length, there is good evidence that scattering is involved rather than absorption. Fluorescence can be detected by observing the spectral composition of the scattered light. That can often be done by interposing in the scattered beam a properly chosen "sharp cut-off" filter (GAGE, 1945) having practically zero transmittance for the scattered light and relatively high transmittance for the fluorescent light. Further, the angular divergence of the transmitted beam must be as small as possible and the cell walls blackened so that scattered and reflected light does not contribute significantly to the measured intensity. The concentration must be so low that multiple scattering can be neglected. DOTY and STEINER (1950) have very thoroughly treated the advantages and limitations of direct turbidity measurements.

2. *Measurement of scattered light*

Although earlier experiments were all carried out by visual or photographic methods of measuring the scattered intensity and there are even now a great many visual turbidimeters, Tyndallmeters and nephelometers in use, nearly all recent instruments employ photoelectric cells and photomultipliers as light detectors. The extreme sensitivity of these tubes has made possible the investigation of much more dilute solutions even in the case of particles with low scattering

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power (small particles, small difference in refractive index between the particle and the solvent). PUTZEYS and BROSTEAUX (1935) were among the first to use photocells for that purpose. One of the very first very satisfactory types of apparatus with a photomultiplier tube was that of ZIMM (1948b). In his arrangement the cell containing the sample is a small thin-walled slightly conical glass bulb, immersed in an outer conical cell containing a liquid of approximately the same refractive index as that of the glass. Thus the multiple reflexions on the glass walls of the sample cell are considerably reduced. The scattered light is received on a photomultiplier tube and the current compared with that of a simple photocell receiving a well known small portion of the primary beam. Thus the influence of intensity fluctuations of the light source is eliminated. BRICE *et al.* (1950) have designed an apparatus* with a six-sided scattering cell for measurements at 0°, 45°, 90° and 135°. The necessary corrections for reflexion, refraction and finite angular spreading that must be made to the experimental measurements, are treated with great thoroughness in the above cited publications. A modification of this type suitable for measurements at high temperature, e.g. for the investigation of polyethylene samples, was designed by MOORE (1956) and by KOBAYASHI *et al.* (1957). HARVEY and CLEVERDON (1954) use a simple cell which differs entirely in design from those currently employed. The metal cell with 4 plane glass windows for observation at 45°, 90° and 135° has blackened walls and light-trap cones so that it is as completely as possible free from stray light although the diameter of the incident beam is rather large. Its disadvantage is the large volume of the cell (216 ml). A semimicro light-scattering apparatus was built by GORING (1953), requiring only 4 ml of the sample and permitting measurements from 37·5° to 135°. A simple universal high sensitivity photometer was designed by OSTER† (1953) with different exchangeable cells permitting measurements at 0° and from 15° to 150°. The instrument of AUGHEY and BAUM (1954) has a very great useful angular range from 0·05° to 140° with an angular resolution of 0·02°, permitting the measurement of large inhomogeneities at small angles as they occur with very large particles. Light intensities varying by a factor of 10^8 are measured through the use of an optical attenuator by a photomultiplier and recorded at different scanning rates. A perfection of the Zimm type instrument is that of WIPPLER and SCHEIBLING (1954). The liquid surrounding the cell with the sample is benzene which has nearly the same refractive index as glass so that all multiple reflexions on the cell walls disappear (Fig. 14).

* The apparatus is now commercially available (Brice-Phoenix Light Scattering Photometer, Phoenix Precision Instr. Co., Philadelphia, Pa., U.S.A.).

† Commercially available as Aminco Light-Scattering Photometer (Amer. Instr. Co., Silver Spring, Md., U.S.A.).

Further the photometers of DEBYE (1946), DOTY *et al.* (1947), SARGE (1948), BIER and NORD (1949b), BLAKER *et al.* (1949), HADOW *et al.* (1949), BISCHOFF and DESREUX (1950), KUNST (1950), DOTY and STEINER (1950), JULLANDER and BRUNE (1950), KERKER and LA MER (1950), HENGSTENBERG (1951), BOSWORTH *et al.* (1952), ATHERTON (1952), GORING and JOHNSON (1952), HALWER *et al.* (1953), OTH *et al.*

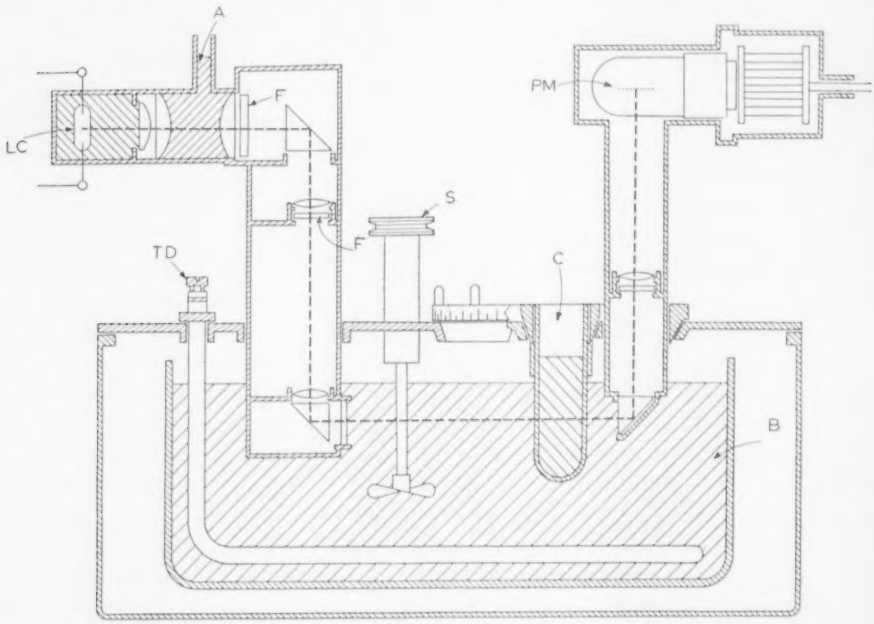

Fig. 14. A modification of the light scattering apparatus of WIPPLER and SCHEIBLING (1954), built in the J. Stefan Institute, Ljubljana. LS, light source; A, absorber of infrared radiation; F, filters; TD, thermostatic device; S, stirrer; C, sample cell; B, benzene bath; PM, photomultiplier tube.

(1953), ZIMM (1953), SEDLAČEK (1953), CANTOW and SCHULZ (1954a), FENDLER *et al.* (1954b), CASASSA and KATZ (1954), TRAP and HERMANS (1954b), BILLMEYER and DE THAN (1955), DUCH and KÜCHLER (1956), TABIBIAN *et al.* (1956), CANTOW* (1957) have to be mentioned, but the list is far from being complete.

Most of the apparatus can also be employed for the measurement of the *depolarization ratio*. The primary beam usually passes through a proper filter which separates the green (5461 Å) or blue (4358 Å) line of the mercury lamp from the continuous background and also from all other lines not used. A polarizer can also be inserted permitting measurements with horizontally or vertically polarized light. By an

* Commercially available from Netheler and Hienz, Hamburg, Germany.

analyser placed in the scattered beam, mostly just in front of the collecting lens of the photocell, the horizontal (H) and vertical component (V) can be measured. Because the photocells are not equally sensitive to light of different polarization direction, a proper correction has to be applied to the measured currents to obtain true intensities.

The *absolute* determination of the reduced intensity R, being of the order of 10^{-5}, is very difficult due to its extreme smallness and is hence very inprecise. Usually one compares the measured values of $R(\theta)$ with those of a well known *standard*, that is either with a low molecular liquid such as benzene, toluene, carbon tetrachloride or carbon disulphide, or with a solid or liquid scatterer with well known turbidity (*relative* determination). There has been a long controversy about the true value of the reduced intensity at 90° (Rayleigh ratio) of the low molecular scattering standards. But all the recent investigations by Sedlaček (1953), Maron and Lou (1954b), Cantow (1956) and Rempp and Benoit (1957) confirm the "high" values of Carr and Zimm (1950) reproduced in Table 1. Carbon disulphide has the disadvantage of becoming noticeably yellow in a relatively short time after exposure to 4358 Å radiation. All the liquids in Table 1 save CCl_4 have large

TABLE 1*

	λ_0(Å)	$R(90°) \times 10^6$	Δ_u	
Benzene 25°C	4358	48·5	0·41	Carr and Zimm (1950)
	5461	16·3	0·40	
Toluene 25°C	4358	49·0	0·47	Cantow (1956)
	5461	17·2	0·45	
CCl_4 30°C	4358	15·8	0·048	Carr and Zimm (1950)
	5461	5·9		
CS_2 25°C	4358	240	0·66	Cantow (1956)
	5461	72·5	0·64	
H_2O 25°C	4358	2·89	0·08	Kraut and Dandliker (1955b)
	5461	1·05		

* The values of Δ_u for CS_2 are higher than 1/2, the highest value for independent particles (see equation 15). The difference is due to the interaction effect of the closely packed molecules of the liquid.

depolarization ratios and hence require the use of the respective depolarization correction factors during instrument calibration.

Instead of calibrating the photometer with a standard low mol. wt. liquid, one may also use a stable colloidal suspension, for instance du Pont Ludox, a vitreous suspension in water (Mommaerts, 1952; Tietze and Neurath, 1952; Oster, 1952; Oth *et al.*, 1953; Maron

and LOU, 1954a; TRAP and HERMANS, 1954b) or a solution of a standard preparation of polystyrene, prepared by the Dow Chemical Co. and distributed by DEBYE and BUECHE of Cornell University, Ithaca, N.Y., U.S.A. (see BRICE *et al.*, 1950; EDSALL *et al.*, 1950), which, however, turned out to be not very stable, or a very pure, many times recrystallized preparation of bovine serum albumin with $M = 75{,}000$ (EDSALL *et al.*, 1950). CANTOW and SCHULZ (1954a) calibrate their photometer with solutions of polymethylmethacrylate of well known mol. wt. and thus avoid the uncertainty in the correction factors of the apparatus. The solution of the standard can be chosen in such a way that the scattering intensity is close enough to the intensity of the unknown sample which has to be investigated. Thus the performance of the apparatus can be checked and the necessary corrections for the reflexions and the refractive index effect easily determined.

Solid blocks of plastics or opal glass of moderate turbidity have been used as working standards (ZIMM, 1948b; BRICE *et al.*, 1950; GORING and JOHNSON, 1952) but their properties may change with temperature and time. Their turbidity and scattering are first determined at the calibrating of the apparatus with one of the above mentioned primary standards. After that all the measurements are referred to them and have only occasionally to be compared again with the less handy primary standard.

The intensity measured by the photocell has to be corrected by some factors in order to obtain the true scattered intensity per ml of the sample. First of all for a strictly parallel incident beam the volume sending light into the measuring equipment is proportional to $1/\sin\theta$ so that the observed value has to be multiplied by $\sin\theta$(1). In most experimental arrangements the scattered light emerges from the sample into air on its way to the photocell. Due to the change of *refractive index* (2) on the walls the divergence of the scattered beam increases. According to HERMANS and LEVINSON (1951) the readings have to be multiplied by n^2 irrespective of the shape of the sample cell. This correction factor was checked by MEIER (1953) for rectangular and conical cells. The *reflexion* (3) of the scattered light on the cell walls depends not only on the refractive index difference but also on the geometry of the cell. By blackening the back of the cell or by inserting an absorbing glass the increase of intensity by reflexion on the back side can be avoided; but the reduction due to the reflexion loss of the beam on its way to the photocell still remains and has to be determined during the calibration of the instrument by a well known standard. This effect is rather disturbing when it shows a marked angle dependence. *Multiple reflexions* on the cell wall have to be avoided either by surrounding the cell with a liquid having the same refractive index as glass (benzene) or by a special shape of the cell and the primary beam

windows. Some of the corrections, i.e. (1) and (2), need not be applied to measurement of dissymmetry because they are identical for symmetric observation in respect to the 90° direction. Their contribution is also very much reduced in the dispersion coefficient.

The *sample* itself may introduce errors by its own absorption, fluorescence and multiple scattering. The colour or *absorption* of the sample attenuates the primary and the scattered beam which can be accounted for by the known absorption coefficient and geometry of the apparatus. Correction factors were derived by PUTZEYS and DORY (1940), KENYON and LA MER (1949), TONNELAT and BATSCH (1950), DOTY and EDSALL (1951), LAUER (1951) and BRICE *et al.* (1953). Errors due to the *fluorescence* can be minimized by choice of a longer wave-length which does not excite fluorescence, or by location of a monochromatic filter between the scattering solution and the photocell; but it can also be accounted for by a proper correction factor due to the fact that the fluorescent light is largely unpolarized whilst the excess scattered light is almost completely polarized, and it is of longer wave-length than the scattered light. By a proper correction factor BRICE *et al.* (1953) were able to measure the scattering with errors of less than 3 per cent even when 80 per cent of the total phototube response was due to fluorescence. In polyvinylbromide in tetrahydrofuran, however, CIFFERI *et al.* (1957, 1958) have found a rather high polarization of the fluorescent light. The proper modification of the correction factor is given by the authors. *Multiple scattering* occurs at higher concentrations of large, strongly scattering particles and can be avoided by increasing the dilution. It hence automatically disappears at the usual extrapolation to zero concentration; but it can be rather disturbing in direct turbidity measurements as, for instance, in calibrating the light scattering apparatus by Ludox colloidal sols of high turbidity (KRAUT and DANDLIKER, 1955a).

The optical and geometrical performance of the scattering photometer may be checked by the measurement of the angular distribution of the radiation of a fluorescent solution at such a concentration that the scattering of the incident light can be neglected. Since the fluorescence of low mol. wt. compounds in solution yields isotropic light, any observed anisotropy is due to a poor geometry, reflexions on the walls or any other type of stray light and has to be removed as far as possible before the instrument can be expected to give reliable informations on light scattering.

3. *Refractive index increment*

The refractive index increment dn/dc due to the solute must be very exactly known. It has to be measured in the linear concentration range because it is wanted in H and K for the infinitely diluted solution.

Special differential refractometers (DEBYE, 1946; BRICE and SPEISER, 1946; BRICE and HALWER, 1951; DEBYE and ANAKER, 1951; SVENSSON and ODENGRIM, 1952; SCHULZ *et al.*, 1952; MARTENS and STUART, 1953) were developed by which the refractive index difference may be measured to about 3×10^{-6}.

4. *Preparation of the samples*

Special attention has to be given to purification of the solutions for any impurities and dust particles may very profoundly influence the intensity and angular distribution of the scattered light. Large dust particles can be easily detected by inspecting the sample in strong side light with a magnifier lens or a small microscope. The detection is the more sensitive the smaller the angle between the illumination and observation direction. Their removal can be achieved by filtration through very fine sintered-glass filters under pressure (THURMOND, 1952) or through bacteriological filters (STEIN and DOTY, 1946), or by centrifugation at high accelerating fields (30,000 *g* and more) provided the solute does not sediment. The most troublesome are microgel particles which resist centrifugation and still pass through filters.

The determination of the scattered intensity from the *pure solvent* which must be subtracted from the reading for the solution has to be carried out with great precision. Any angular dissymmetry is a sure indication of dust particles in the solvent which must be carefully removed by filtration or centrifugation. The situation with water is usually worse than with other solvents, the readings being generally considerably higher than would be expected from the tabulated reduced intensity of the water itself. This stray light contribution has, of course, also to be subtracted from the reading for the solution, but since it varies from sample to sample every effort must be made to reduce it to a minimum, so that the correction becomes negligible.

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VISUAL PIGMENTS IN MAN AND ANIMALS AND THEIR RELATION TO SEEING

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5

VISUAL PIGMENTS IN MAN AND ANIMALS AND THEIR RELATION TO SEEING

W. A. H. Rushton

I. Introduction

1. *The importance of visual pigments*

Light has so widespread an effect upon living organisms that it is quite the exception to find a species which is not equipped with some mechanism of response. Apart from the particular case of photosynthesis in green vegetation, there is the widespread growth of plants towards the light, and the far more complicated reactions of most animals to brightness and shadows. This may culminate in the appreciation of form, of movement, of colour and of the degree and plane in which light is polarized. But if light is to have any effect upon matter at all it must be absorbed by it, and since any substance which absorbs light is a pigment, it is only by falling upon a pigment that light can produce its biological effect.

Now most pigments are stable chemical substances like the pigments in a paintbox. They absorb light of certain wave-lengths and reflect or transmit the rest (which give the characteristic appearance to the paint) but no change results except for the conversion of the minute light energy into heat which thus raises slightly the temperature of the pigment. There is, however a comparatively small number of pigments which behave like the silver bromide of a photographic film and undergo chemical change as a result of the absorption of light. The value of these *photosensitive* pigments is that they do not allow the light energy to become degraded into heat where it is useless as the starting point of a biological reaction. On the contrary, in those visual mechanisms which have been studied, the light quanta absorbed appear to trigger off far greater quantities of energy stored in the retina and this results in a measureable electric change, which probably initiates impulses in the optic nerve going to the brain.

The importance of the visual pigments then lies in the fact that they, and they alone, catch the light quanta which form the starting point of the visual process, and colours which they will not absorb will not be seen.

It is therefore worth asking the question "What photo-sensitive pigments are present in the living retina, and how are they related to vision?" The present attempt to answer this will be confined to the vision of vertebrates.

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2. *The discovery of visual pigments*

The first clear objective demonstration that the retina contains a photosensitive pigment was made in 1876 by BOLL, who saw that a frog's retina removed in very dim light was strongly rose-coloured, and that this colour when observed in good light against a white background quickly faded. The observation was immediately seized upon by KÜHNE with such enterprise and energy, that in the next few years he and his colleagues discovered nearly everything that could be found with the resources then available (KÜHNE, 1877–8, 1878–82).

In particular, the rose-coloured substance, *rhodopsin* or "visual purple" was seen to be in the rods but not in the cones or anywhere else; it was bleached to a yellow and finally a colourless product, but would regenerate if the retina were left in the eye-cup in contact (as usual) with its black lining of pigment epithelium.

KÜHNE also obtained a clear solution of rhodopsin by treating the retina with bile, and he satisfied himself that the changes which occurred upon exposure to light were the same in bile solution as in the intact retina, but in the solution, where there was no pigment epithelium, there was no regeneration.

A clear solution holds for the chemist the promise of purification, isolation and analytical treatment, and by far the most extensive and most important contributions have been made with extracts in bile or (since TANSLEY in 1931) in digitonin. We shall therefore proceed at once to a somewhat brief and over-simplified account of this work with the intention not of tracing the development of the subject but of summarizing the present rather complex position, particularly in relation to the physiology of seeing. The reader who desires a more detailed and authoritative account of the chemical aspects of visual pigments in solution is referred to reviews by WALD (1953), COLLINS (1954) and to the recent book by DARTNALL (1957a).

II. VISUAL PIGMENTS IN SOLUTION

1. *Reactions in light and darkness*

If the rods of frogs, cattle or many other vertebrates are extracted in digitonin, a clear solution is obtained. Its absorption spectrum has been accurately measured by several authorities who agree, and the curve is shown in Fig. 1 (circles). If strong light falls upon the solution, bleaching will occur at a rate proportional to the number of quanta per second which are absorbed. This will depend upon the wave-length of the light and, as is seen from Fig. 1 (crosses from SCHNEIDER *et al.*, 1939), lights which are more readily absorbed are precisely to that extent more effective in bleaching.

Extinction, absorption, action, and difference spectra. The absorption of an extract is measured by placing it in a small glass (or quartz) trough with good parallel faces and passing through it a parallel

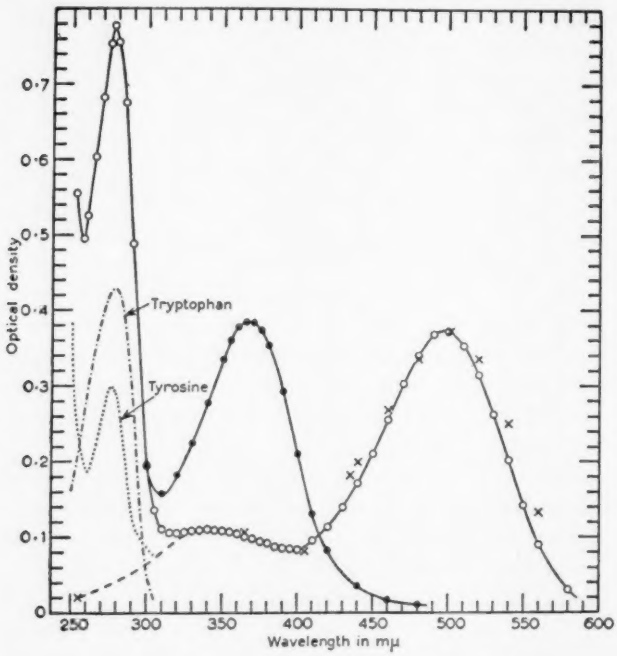

Fig. 1. Absorption spectrum of rhodopsin. Circles, extinction spectrum before bleaching; dots after bleaching; crosses, action spectrum (from DARTNALL, 1957b, after COLLINS *et al.*, 1952).

beam of monochromatic light not strong enough to bleach appreciably. According to Beer's and Lambert's laws which apply to extracts of visual pigments

$$\log_e I_i/I_t = cl\alpha_\lambda \qquad \ldots.(1)$$

where I_i, I_t are respectively the light intensities incident upon the pigment solution, and transmitted through it, c is the number of chromophores of pigment per ml, l the length of the optical path through the solution and α_λ the extinction coefficient per chromophore for the wavelength, λ, used.

Now the value of I_i/I_t is easily obtained by substituting for the trough of pigment, a control trough containing only (transparent) digitonin solution. The transmitted light in each case is allowed to fall upon a photocell and the ratio of the electric outputs will be I_i/I_t. In this way the value of $cl\alpha_\lambda$ can be measured for various

wavelengths, and the curve so obtained, the *extinction spectrum* (circles Fig. 1) is seen to be unaffected by the concentration of pigment used or the dimensions of the trough employed, except for the scaling of ordinates. The extinction spectrum thus represents a molecular property of the pigment and is independent of the conditions of measurement.

The effect of light upon a photosensitive pigment, however, depends upon how many quanta are *absorbed*. The fraction X of incident light which is absorbed will clearly be that which is not transmitted, hence $X = 1 - I_t/I_1$, which substituted in (1) gives

$$\begin{aligned} cl\alpha_\lambda &= -\log_e (1 - X) \\ &= X + X^2/2 + \ldots \end{aligned} \qquad \ldots . (2)$$

The *absorption spectrum* therefore coincides with the extinction spectrum for small densities of pigment such as are commonly found in extracts, and the terms are often used interchangeably in this type of work. But as we shall see later in the living eye, X may attain a value of 80 per cent or more and the absorption spectrum (Fig. 7 dotted curve) diverges notably from the extinction spectrum (Fig. 7 full curve). The extinction spectrum is, however, the key to the absorption curve for any known pigment concentration, since it is clear from (2) that if the relative values of α_λ are known for all wavelengths, and the absolute value of X known for one wavelength, then X may be calculated for all others.

Since a photosensitive pigment breaks down by an amount proportional to the number of quanta absorbed, the absorption spectrum may be measured by comparing the quantum energy of different incident lights all adjusted to bleach the same amount. Such a comparison is called the *action spectrum* and is represented by crosses in Fig. 1 where it is seen that over the visible part of the spectrum the action spectrum of rhodopsin coincides well with its absorption spectrum which is here weak enough to coincide with the extinction spectrum (circles). The action spectrum has not often been measured upon pigment extracts, but it presumably underlies most of the determinations of spectral sensitivity in man and animals.

There is a serious practical difficulty in obtaining a pure extinction spectrum. It is hard to avoid the extract being contaminated with traces of impurity whose extinction will be added to that of the visual pigment. If the impurities are photostable (as is usually the case) they will contribute to the measured extinction equally before and after exposing the solution to a strong light which will bleach the pigment. So the subtraction of these two extinction spectra will

give a *difference spectrum* from which the stable impurities have been entirely eliminated. This no longer gives the extinction or absorption of the original visual pigment, but it is a function of that pigment only, namely the extinction of the pigment *minus* that of photo-products. The curve would be obtained from Fig. 1 by subtracting the ordinates represented by dots from those represented by circles. It is seen that the difference spectrum becomes negative at short wavelengths and is substantially the same as the extinction spectrum for wave-lengths greater than the extinction maximum of the visual pigment. Fig. 3 shows the difference spectra plotted directly for a number of visual pigments.

The immediate effect of light absorption is a chemical change which does not greatly alter the absorption spectrum, but the new compound (lumirhodopsin) is unstable so that a series of chemical changes follow which depend upon the temperature but not upon the presence of further light (WALD *et al.*, 1950, extending the analysis of BRODA and GOODEVE, 1941). These changes go through a transient orange stage to a stable yellow compound (dots Fig. 1) whose colour depends upon the *p*H of the extract and was therefore named by LYTHGOE (1937), "Indicator yellow." If this solution is extracted with chloroform or petroleum ether (WALD, 1938,) a carotenoid, *retinene* (WALD, 1935) is obtained which BALL *et al.* (1948) showed to be the aldehyde of vitamin A. Rhodopsin is therefore a compound of vitamin A aldehyde and protein, and the question arises as to whether the synthesis can be performed *in vitro* from these ingredients.

Vitamin A can be oxidized to retinene either by inorganic oxidizing agents such as MnO_2 used by BALL *et al.* (1948) or by the widespread enzyme *alcohol dehydrogenase* (BLISS, 1949). By exposing the retinene so formed to *scotopsin*, the protein extracted from rods, HUBBARD and WALD (1951) were able to convert vitamin A to rhodopsin whose properties were indistinguishable from those of a normal extract from the retina. The synthesis may be represented

$$\text{Vit. A} \underset{\text{DPN}}{\overset{\text{DPN}-\text{H}_2}{\rightleftharpoons}} \text{Retinene} \underset{-}{\overset{+\text{opsin}}{\rightleftharpoons}} \text{Rhodopsin}$$

Now vitamin A (and retinene) contains in its structure several double bond linkages; consequently different isomers can exist depending upon whether the linkage is *cis* or *trans* at each double bond. It turns out that only one configuration of retinene is suitable for the regeneration of rhodopsin (HUBBARD and WALD, 1952), namely the 11-*cis* isomer represented in Fig. 2*b*. It is combined with protein in the rhodopsin molecule and it might be supposed that this would be the form released again when rhodopsin is bleached. However, the retinene extracted after full bleaching turns out to be the most stable all-*trans*

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configuration, Fig. 2*a*. Thus it appears that one very important feature in the regeneration of rhodopsin is the preliminary isomerization of the useless all-*trans* to the necessary 11-*cis* configuration of retinene or vitamin A.

The nature of isomerization during bleaching is elucidated by a striking observation of COLLINS and MORTON (1950). A rhodopsin solution (in glycerol) was cooled to — 70°C and there exposed to an excess of light so that the photic reaction proceeded to the full but the thermal reactions were arrested at the initial orange stage by the low temperature. The extract was then placed in the dark, brought to

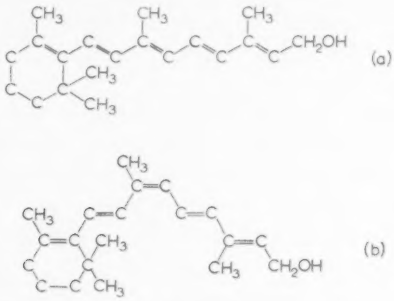

Fig. 2.

room temperature and then analysed spectroscopically. It was found that half of the rhodopsin had broken down to indicator yellow in the ordinary way, but half had regenerated to rhodopsin or rather something very like it which they called isorhodopsin. If the whole procedure was repeated upon the resulting mixture, the indicator yellow remained unchanged but the isorhodopsin after being "bleached" to orange in the light and cold, was changed in the dark at room temperature so that half of *that* went to indicator yellow and half was reconverted to isorhodopsin.

A probable explanation is that in the light, the retinene moiety of the rhodopsin is isomerized into many forms, and during the dark reactions, the isomers unsuitable for regeneration decompose to indicator yellow leaving rhodopsin and related isomeric forms (HUBBARD and KROPF, 1958). During prolonged lighting at room temperature these cycles are rapidly occurring so that in the end, only the inert all-*trans* form remains, which needs isomerization with the expenditure of energy to be converted into the 11-*cis* form necessary for regeneration.

The nature of isomerization *in vitro* has been studied in a very simplified and purified system by HUBBARD (1956). There was extracted from the retinas of cattle an enzyme, *retinene isomerase*, which would act upon retinene but not upon vitamin A, and which

needed energy supplied in the form of irradiation to give a significant yield of 11-*cis*. But since irradiation of retinene appears to play no part in the regeneration of rhodopsin in the living human eye (RUSHTON, 1957b), it is likely that *in vivo* the energy is supplied chemically from ingredients absent in the pure system of HUBBARD, but present in the more complex conditions of COLLINS *et al.* (1954).

2. *The general properties of visual pigments*

Rhodopsin is the visual pigment easiest to extract in quantity and hence most work on extracts have been concerned with it. Nevertheless a very considerable body of work has been done upon other pigments with the satisfying result that nearly all those studied conform to the same pattern. We shall here pass over individual details and simply indicate this general scheme.

2.1. *The retinenes.* All vertebrate visual pigments resemble rhodopsin in being compounded of a carotenoid, the *retinene*, and a protein, the *opsin*. Two vitamins A are known called A_1 and A_2, and from these vitamins two retinenes, two visual pigments and their sets of photoproducts are found exhibiting analogous properties but with the A_2 compounds absorbing at wave-lengths some 30 mμ greater than the A_1 analogues.

Retinene$_2$ is found in the eyes of fish hatched in fresh water and some amphibia. Their rod pigment is called not rhodopsin$_2$ but *porphyropsin* (= visual violet). A fascinating story can be written (WALD, 1945) on the role of carotenoids (such as retinene compounds) in directing the movements of animals and vegetables, but in detail there is still much to be learnt, and even among vertebrates we are ignorant of the real factors which determine whether A_1 or A_2 or a mixture will be found in the eye.

So far, then, as the retinene components are concerned, all visual pigments appear to be compounded of the 11-*cis* isomer of either retinene$_1$ or retinene$_2$. The alternative is sharp and substitution produces the well-defined spectral displacement of 30 mμ or so. What is the effect of change in the *opsin*, the protein component of the pigment?

2.2. *The opsins.* By far the most striking answer to this was provided when WALD *et al.* (1955) repeated *in vitro* the synthesis of rhodopsin indicated above (p. 244); but instead of using proteins extracted from rods, they used proteins from the cones of fowls. The chemical system was identical except for the protein, but a new photosensitive pigment, *iodopsin*, was obtained with the absorption spectrum shifted some 60 mμ further towards the red than rhodopsin, and with far faster kinetics of regeneration. An analogous A_2 pigment *cyanopsin* was synthesized from vitamin A_2 using the same *photopsin* from fowls'

cones (WALD *et al.*, 1953). It is therefore clear that a substantial alteration in the pigment can be produced by change in the carotenoid, and an even greater alteration by a change in the protein from the *scotopsin* of the rods to the *photopsin* of the cones.

When we observe that a spectral shift of about 60 mμ may be produced simply by changing the protein component from scotopsin

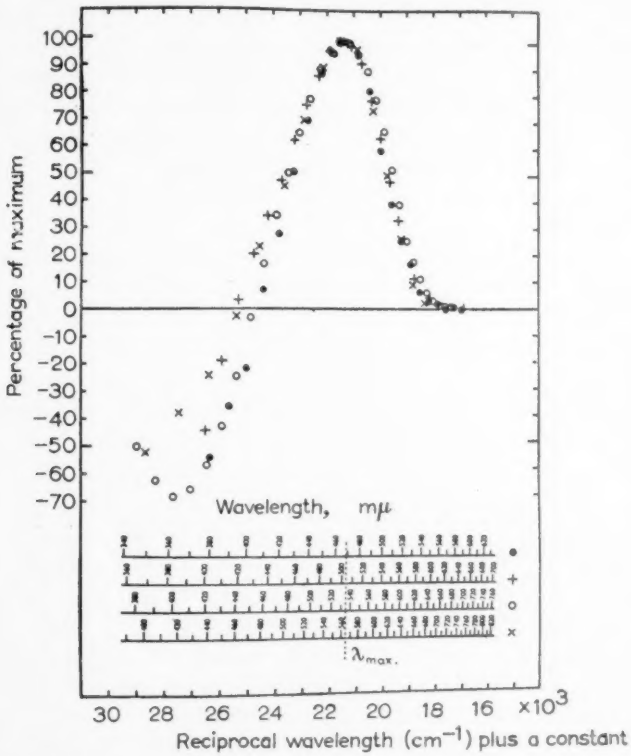

Fig. 3. The difference spectra of 4 visual pigments plotted as function of wave frequency and displaced horizontally so that the maxima coincide (from DARTNALL, 1952).

to photopsin, and when we consider the great complexity of protein structure and the general phenomenon of animal specificity, it is remarkable how nearly identical are the rhodopsins from frogs and men. It would not therefore be surprising if minor or even major changes in the absorption spectrum were found in various species resulting from modification of protein structure, and no doubt this largely accounts for the very great variety in detail of "rhodopsins" and of other visual pigments which are being found as investigations reach deep sea fish

and other less accessible species (DENTON and WARREN, 1956; MUNZ, 1958; also DARTNALL, 1952, 1955; CRESCITELLI and DARTNALL, 1954).

2.3. *The shape of the absorption spectrum.* It is usual to plot absorption curves against a uniform scale of *wave-lengths*, but from considerations of physical chemistry a uniform scale of wave-numbers (= reciprocal wave-lengths) would be more satisfactory. DARTNALL (1952) has pointed out that when plotted in this way the spectral absorption curves of all the visual pigments appear to have nearly the same shape and differ only by their position along the horizontal axis of wave-frequencies. Fig. 3 shows how nearly coincident are the difference spectra of 4 different pigments whose maxima are at 470, 500, 533 and 565 mμ if displaced horizontally so that their maxima coincide. This close agreement seems to apply to all the visual pigment extracts of vertebrates whether from A_1 or A_2, with proteins from rods or cones.

2.4. *The rate of bleaching.* If an extract is not quite exceptionally concentrated, the amount of a given light which must fall upon it in order to bleach away (say) half will be a quantity which is independent of the concentration of the extract. For, a second extract n times as concentrated as the first will absorb the incident light at n times the rate, but molecules are n times as numerous, hence the *proportion* of molecules bleached at any time will be the same in the two samples.

Pigments compared in this way might conceivably require very different light energies to fall upon them in order to produce half-bleaching. But DARTNALL's (1957b) measurements upon pigments from the frog and 4 fish (maximum absorption varying from 487 to 533 mμ) show great similarity in the bleaching rate. When each pigment was exposed to light whose wave-length corresponded to the absorption maximum of the pigment it was found that nearly the same quantity of energy was needed for half-bleaching. It is probable that these are all rod pigments. We have no comparable data from cone pigments.

Work upon visual pigments in solution, therefore, has given an accurate and coherent body of knowledge which continues to grow rapidly. Our aim is to relate this knowledge to the physiology of seeing.

III. VISUAL PIGMENTS IN THE RODS

1. *Organization*

In extracts, the molecules of visual pigments are distributed at random: in the rods they are *organized*. They are collected into separate rods and concentrated in their outer segments, and orientated so that they all lie in planes normal to the rod's axis. This was discovered by SCHMIDT (1937) who observed detached rods on a microscope slide in polarized light and saw that they lost their purple colour when viewed

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by light whose electric vector was parallel to the axis of the rod. DENTON (1954) saw this even more strikingly in the excised whole retina by folding it upon a slide and looking through the "forest" of rods at the fold, using light polarized either vertically or horizontally. Such a molecular arrangement is the most efficient one to absorb light passing axially down the rods, but it constitutes an improvement over the random arrangement only in the ratio 3:2.

No doubt, related to this orientation is the fine structure of the rod outer segment revealed by the electron microscope (SJÖSTRAND, 1949) as a pile of some thousand discs. Cones have a similar structure though no pigment has yet been directly seen in a cone to test the dichroism. A rather similar structure, moreover, is found in the chloroplast of plants, and it is likely that molecular organization of a high order is involved. This is hardly to secure the mere 3/2 improvement in quantum catching power; rather is it concerned with the efficient use of the high potential of light energy, in order that a single quantum may trigger some response from a rod. For though it requires several quanta to produce a threshold sensation (see review by PIRENNE, 1956) each single quantum in general will fall upon a different rod, and hence must be sufficient to excite it.

We have no knowledge of the nature of this excitation nor of the essential change in the rhodopsin molecule which produces it. The most enlightening study that has been made upon the generation of impulses in an optic nerve is that of HARTLINE and colleagues upon the invertebrate, *Limulus* (HARTLINE *et al.*, 1952). Nerve impulses are preceded and accompanied by a slow potential change (the ommatidial action-potential) which probably is the actual stimulus which generates them. Between the flash and the start of this slow potential there is silence.

It is a large jump from the eye of the arthropod to that of the vertebrate, but it is tempting to suppose that the electro-retinogram (E.R.G.) may here play a part similar to the ommatidial action-potential in *Limulus*. Like it, the E.R.G. is the earliest detectable change following photolysis and both slow electric changes are found to depend similarly in size upon the intensity of the light flash and upon the extent of light or dark adaptation.

Though we do not know in what way the bleaching of a visual pigment generates the E.R.G. or impulses in the optic nerves, we do know that quanta which are not caught will not be seen. So it might be supposed that wavelengths of light best seen will be those best absorbed by the visual pigments concerned. We shall therefore enquire in the case of man and some other vertebrates what is the absorption spectrum of the pigment of the rods, and how well does this agree with the spectral sensitivity of the animal when using rod vision.

2. *Spectral sensitivity*

2.1. *Man.* MAX SCHULTZE (1866) studied the microscopic anatomy of the retina in a wide variety of vertebrate types. He found that animals whose retinas contained mainly cones were active only in day-time, whereas those containing mainly rods were predominantly nocturnal in habit. When therefore 10 years later BOLL and KÜHNE saw rhodopsin in the rods and not in the cones it was natural to associate this pigment with twilight vision. It has long been known that in twilight (below the brightness of a landscape lit by the half moon) visible objects appear without colour, red objects appear black and

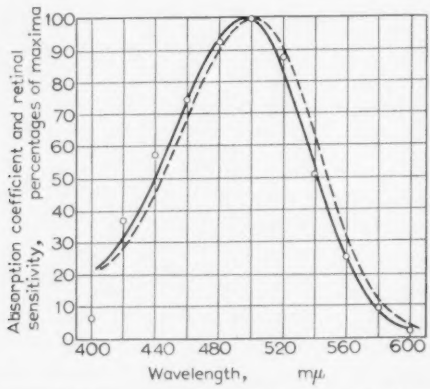

Fig. 4. Circles, corrected human scotopic spectral sensitivity curve. Full line, absorption spectrum of human rhodopsin. Broken line, absorption spectrum of frog's rhodopsin (from CRESCITELLI and DARTNALL, 1953).

nothing can be seen with the central spot of the retina—the *fovea centralis*—which contains cones only, though this is the best part of the retina in daylight. That is precisely what would be expected if in twilight vision only the rods were sufficiently sensitive and if the rhodopsin they contain was the only visual pigment involved. For with only one visual pigment in operation, vision must be monochromatic and since rhodopsin does not absorb red light (Fig. 1) it cannot detect it.

This last relation can be expressed more exactly. The spectral sensitivity may be defined at each wavelength as the reciprocal of the *threshold* light energy at that wavelength (where energy should be expressed in quanta, see DARTNALL and GOODEVE, 1937). Then if rhodopsin is the sole visual pigment in twilight vision, the scotopic (= twilight) spectral sensitivity curve should coincide with the rhodopsin absorption curve, which itself coincides with the rhodopsin action spectrum as we saw in Fig. 1.

Fig. 4 shows the absorption spectrum for human rhodopsin

(CRESCITELLI and DARTNALL, 1953) together with the scotopic spectral sensitivity curve (circles) from CRAWFORD (1949) after correcting for the fact that the human lens is slightly coloured. This accurate correspondence which confirms earlier work (KÖNIG, 1894; TRENDELENBURG, 1904; HECHT and WILLIAMS, 1922) gives great strength to the belief that rhodopsin alone constitutes the visual pigment in human twilight vision.

2.2. *Mammals.* It is important to be able to make a similar comparison between pigment absorption and sensitivity in animals where some index other than sensation must be employed. If it is known

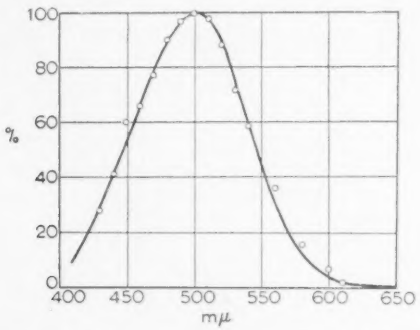

Fig. 5. Comparison of the rhodopsin absorption curve (LYTHGOE) with the scotopic sensitivity of various vertebrate eyes deprived of lens and cornea and tested by GRANIT's microelectrode technique (from GRANIT, 1947).

that only one visual pigment is involved, then *any* response to light which is regularly repeatable may be employed as an index of sensitivity and the E.R.G. (CHAFFEE and HAMPSON, 1924), discharges from the optic nerve (GRANIT, 1941a) or some behavioural response (BROWN, 1936) have often been used.

It is essential to find for each wave-length what is the intensity of light which produces *some fixed response*. The reciprocal of this intensity plotted against wave-length should correspond with the absorption spectrum of the visual pigment if there is only one pigment involved. Often it is technically easier to measure the size of response resulting from a fixed light energy, but this relation is useless for quantitative work unless the calibration curve of response size versus energy is known, from which the essential relation (above) may be deduced.

Whatever index is used, all the work has confirmed the relation stated above for man. Fig. 5, taken from GRANIT's book (1947, p. 269) shows as circles the pooled results of sensitivity measured by microelectrode records from single ganglion cells. They correspond to the reciprocal quantum energy for threshold optic nerve discharges

investigated in cats, rats, guinea pigs and frogs. The points lie accurately upon LYTHGOE's (1937) extinction curve of frog's rhodopsin.

These results are typical of a great mass of experiment all of which confirms the close relation between twilight sensitivity and rhodopsin absorption in the mammal. Those which have twilight vision have rods, and the rods contain rhodopsin and that only. In twilight vision, only rods appear to be used, for the spectral sensitivity corresponds rather exactly to the extinction spectrum of rhodopsin. Moreover, since it is the extinction spectrum of a rhodopsin *extract* that is compared, the disorganization and change of molecular state involved in extraction cannot have changed much the molecular spectral sensitivity.

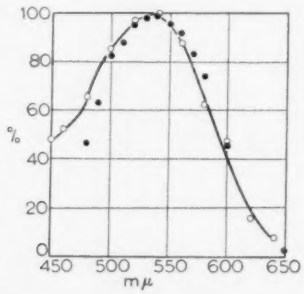

Fig. 6. Comparison of the porphyropsin absorption curve (dots) with the scotopic sensitivity in the tench (circles) obtained by GRANIT's microelectrode technique from 6 fish (from GRANIT, 1941b).

2.3. *Freshwater fish.* It was stated above (Section II, 2.1) that in freshwater fish the rod pigment is derived from vitamin A_2 and is called porphyropsin. This has an absorption curve shifted some 30 mμ towards the red as compared with rhodopsin. Is the sensitivity curve of such fish shifted correspondingly? Only a small amount of evidence is available, but this seems quite definite. GRUNDFEST (1932) studied the movement of the live sun-fish in response to a very dim moving visual stimulus and found that the fish was most sensitive to 540 mμ and could see further into the red than he could himself.

GRANIT (1941b) went further and by leading from retinal ganglion cells obtained a sensitivity curve of the tench which corresponded well with the absorption curve of porphyropsin as may be seen from Fig. 6.

These rather scanty results on spectral sensitivity in freshwater fish fit the porphyropsin picture so nicely that further investigation appears to lack point. However, DARTNALL (1952, 1955) and CRESCITELLI and DARTNALL (1954) have extracted from the eyes of the tench, carp and bleak various photosensitive pigments whose maxima lie at wave-lengths 467, 510, 523, 533 and 550 mμ, and it would certainly be interesting to get sensitivity measurements corresponding to some of these pigments,

but it might not be easy. For, as we shall see, when more than one visual pigment is present, spectral sensitivity may become hard to interpret.

2.4. *Frogs.* From the very beginnings of experimental physiology frogs have served us well, and, amongst other parts, their eyes have been studied very extensively. They have two outstanding advantages. The rods are large and exceptionally rich in rhodopsin: the retina is so independent of its blood supply that regeneration of bleached rhodopsin and even brisk nerve activity in the eye will continue for some hours after it has been removed from the body. Against this stand two

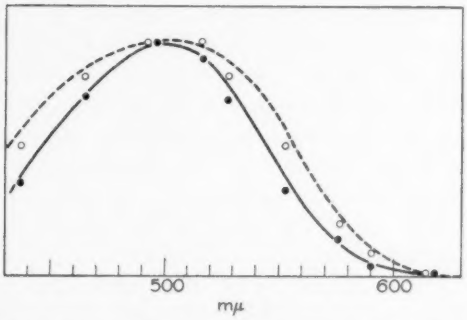

Fig. 7. Spectral sensitivity and rhodopsin absorption in the frog. Full curve, rhodopsin absorption in dilute solution; dotted curve, rhodopsin absorption spectrum when maximum density is 0·7. Dots, spectral sensitivity (single ganglion cell) after 1½ hr dark adaptation; circles after 24 hr dark adaptation (from DONNER and RUSHTON, 1959c).

awkward features. There is great movement of black pigment granules up between the rods in light adaptation, with some movement of the rods and cones themselves: the *rod* population is not homogeneous but consists of about 90 per cent rhodopsin rods and 10 per cent "grass-green" rods. The high density of rhodopsin and the mixed rod population calls for a modification in the simple comparison between sensitivity and the extinction curve of a visual pigment and forms an introduction to the more complex conditions met in cone vision.

Fig. 7 shows the spectral sensitivity curve obtained by GRANIT's technique upon a frog's eye which had been excised and dissected in daylight and left for 90 min in the dark (from DONNER and RUSHTON, 1958c). The black circles fit the (continuous) extinction curve of rhodopsin extracts as they do in Figs. 4 and 5. But so good a fit was hardly to be expected if the rhodopsin density in the rods was as great as that found by DENTON and WYLLIE (1955). They kept frogs in the dark for a day or more, removed the retinas in dim red light and floated them, rods upward, in a shallow saline trough upon a

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microscope stage. A photomicrograph taken in green light is shown in Plate I (*a*) before bleaching and I (*c*) after bleaching. Initially the rhodopsin rods absorb most of the green light and appear dark; after bleaching they become transparent. By calibrating the photographic processes the density of rhodopsin can thus be measured in a single rod. It amounts at maximum to about 0·7 log units which means that some 80 per cent of green light incident upon the rods is absorbed. Now with so dense a mass of pigment it must follow that the more deeply situated molecules will "see" the light only through the intense purple screen of pigment lying superficially. Therefore "self-screening" will cause the *action* spectrum of a dense pigment to differ from its extinction spectrum. The relation is easy to work out (Hecht *et al.*, 1942) and for the density of 0·7 found in the frog's rods after 24 hr in the dark, the action spectrum should be the dotted curve of Fig. 7.

Now the black circles of Fig. 7 which do not fit the dotted curve were not obtained from a retina which had been 24 hr in the dark, but only 90 min. Direct measurements of the rhodopsin after 90 min of regeneration showed a density of only 0·2 (confirming Zewi, 1939) so self-screening is negligible and the sensitivity *should* correspond to the continuous curve. When, however, the sensitivity was repeated upon eyes dissected in red light after 24 hr in the dark, the white circles were obtained. And these are seen to accord better with the action spectrum expected from a rhodopsin density of 0·7. Thus even when only one visual pigment is involved it is not sufficient to consider simply its extinction curve and to expect that spectral sensitivity will coincide. The density of the pigment in the rod itself must also be taken into account and allowance made for self-screening.

When, however, more than one visual pigment is present the situation may become quite difficult. In Plate I (*a*) it is seen that among rods, most of which appear dark in green light, there are a few which appear white. These are the "grass-green" rods of Boll and Kühne. Seen in white light they are green before bleaching and grey after, and as seen in Plate I (*c*) they never become so transparent as the rhodopsin rods. Denton and Wyllie (1955) found these rods to be bleached only by blue light and in Plate I (*b*) it is seen that they absorb blue much more strongly than does rhodopsin. Dartnall (1957a, p. 191) describes a fraction of the extract of frog's retina obtained by partial bleaching which might well correspond to the visual pigment concerned.

The fact that the threshold sensitivity curves of Fig. 7 fit rhodopsin so closely indicates that vision by the green rods is less sensitive than by the purple ones, so at absolute threshold rhodopsin is the only pigment involved. This does not necessarily mean that a green rod is less effective than a purple rod in catching quanta. The great signal to

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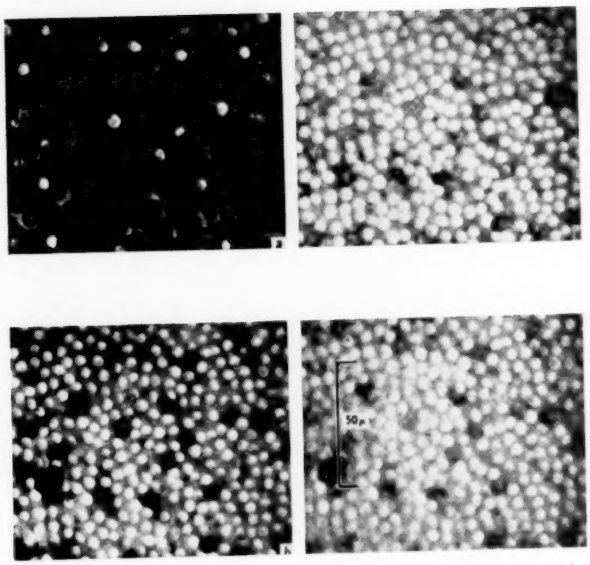

Plate I. Photomicrographs, all of the same part of one frog's retina taken (*a*) in green (*b*) in blue light before bleaching; (*c*) in green (*d*) in blue light after bleaching (from DENTON and WYLLIE, 1955).

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noise sensitivity of rhodopsin rods is partly due to their organization in large, close-packed groups with nerve connections converging upon single optic nerve fibres. The sparse and scattered green rods cannot enjoy such a social organization and hence though they might have equal quantum-catching power they would only become effective at higher light intensities.

This indeed was found to be the case by GRANIT (1942a) and has been confirmed and extended by DONNER and RUSHTON (1958b). Fig. 8 shows a spectral sensitivity curve at moderate illumination determined

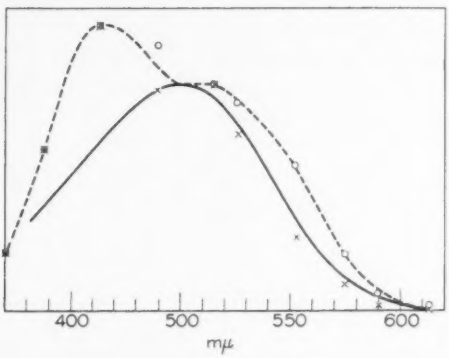

Fig. 8. Continuous curve, spectral extinction of frog's rhodopsin: circles, spectral sensitivity (single ganglion cell) to light falling perpendicularly upon the retina; crosses, sensitivity to light falling obliquely (from DONNER and RUSHTON, 1958).

by recording from a ganglion cell with GRANIT's technique. The continuous curve is the extinction spectrum of rhodopsin, and the sensitivity (circles) which had coincided so well with this in dark adaptation (Fig. 7) now exhibits humps of increased sensitivity both in the yellow and in the blue. In order to test whether *cones* are involved, use was made of the discovery of STILES and CRAWFORD (1933) that if a light falls obliquely upon the human retina it becomes much less effective in exciting the cones but it remains equally effective in exciting the rods (FLAMANT and STILES, 1948). This retinal directional effect upon cones but not upon rods was shown to apply to the retina of the excised frog's eye (DONNER and RUSHTON, 1956) and so it may be used to test for the presence of cones in the sensitivity curve of Fig. 8. When the measurements were repeated using oblique light, results were as shown by the crosses. The hump in the yellow disappears and hence was due to cones; that in the blue persists and hence was due to rods. The blue-sensitive rods are clearly not the rhodopsin rods, and thus presumably are the "grass-green" rods. This interpretation agrees with DENTON and WYLLIE's observation that these rods

are sensitive to blue light and not to green, for DONNER and RUSHTON found that the blue hump was unaffected by adaptation to strong green light, but a blue light which had little effect upon rhodopsin removed the blue hump entirely.

Can we find from a combined sensitivity curve such as that of Fig. 8 what is the sensitivity of the blue receptor? Certainly we may subtract the known rhodopsin curve from the combined curve and obtain a remainder that would not have existed but for the blue receptor. We can do this subtraction of ordinates equally well if the quantity plotted is not sensitivity but log sensitivity, or any other function of sensitivity. And all these will give different answers. The only theoretical justification for the subtraction of curves would be the belief that some known function of the light intensity produces a response from the green rods which simply adds to a similar response from the purple rods to reach a critical threshold level. In that case a spectral plot of light energies scaled according to the known function will represent the response curves which *ex hypothesi* are additive.

This belief may well be true, but it is difficult to devise an experiment which will either disprove or justify it, nor is there any agreement as to the function of light intensity which should be employed. In this situation, the extraction of separate receptor sensitivities from a combined sensitivity curve has no easy solution.

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3. *Rhodopsin bleaching and rod function*

Though our knowledge is almost entirely lacking concerning several important stages in the transformation both of the energy from absorbed quanta into patterns of nerve impulses, and of these in turn into their sensory correlates, some courageous attempts have been made to bridge the gap by hypotheses which seem basically plausible and quantitatively acceptable. Of these none has received wider support and stimulated more research than the photochemical theory developed with such power by HECHT (1937).

The facts to be explained are the variations which occur in threshold, discrimination, etc. when the eye is adapted to various levels of illumination. In principle the explanation derives from the fact that visual pigments are destroyed by light and resynthesized from the photolytic products, etc. and thus attain an equilibrium level of partial bleaching under any steady light. The stronger the steady light the lower the level of unbleached pigment which can be maintained. The fundamental postulate of the photochemical theory is that the variations of threshold, discrimination, etc. which occur are so dependent upon the equilibrium level of pigment attained, that other factors may be neglected. This postulate was supported by some accurate experiments which agreed well with predictions from the photochemical theory.

There are two main types of experiment which have been widely interpreted in terms of photochemistry—the change of threshold which occurs during dark adaptation, and the dependence of acuity, intensity discrimination, flicker, etc. upon the level of light adaptation. The two interpretations run together in a somewhat uneasy harness.

3.1. *Rhodopsin changes in dark adaptation.* It is a familiar fact that after exposure to bright or indeed only moderate lights, the sensitivity of the eye for dim lights is very much poorer immediately than after a stay of some 30 min in the dark. This improvement in sensitivity is

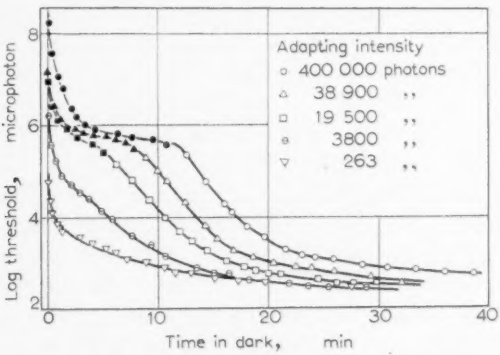

Fig. 9. The course of dark adaptation following 2 min light adaptation to the intensity shown. Filled in symbols where the violet test light appeared violet, i.e. cones active (from HECHT *et al.*, 1937).

known as dark adaptation and it has been measured countless times by determining at various moments during the stay in the dark what is the weakest light visible according to some specified criterion.

Fig. 9 shows results from HECHT *et al.* (1937) where log threshold is plotted against the time in the dark, following 2 min of light adaptation at various intensities.

Intensities are given, as they should be, in photons (= trolands). This is a unit of *retinal* illumination and takes into account the fact that more light will fall upon the retina for given external illumination if the pupil is more dilated. One photon is the retinal illumination when 0·1 millilambert (mL) is viewed through a 2 mm diameter pupil. And 0·1 mL is the brightness of a white screen illuminated by a candle at a distance of 1 m.

As KOHLRAUSCH (1922) first pointed out, the curves obtained after adaptation to a strong light show a kink, the first part corresponding to cone function, the second part to rods. In Fig. 9 the points are filled in black where the violet test light appeared violet in hue; at other points the light was colourless. In this way the fact that cones but not

rods can appreciate colour was used to confirm the cone/rod interpretation of the kinked curve.

These experiments, with minor modifications, have been repeated times without number and nearly always interpreted in terms of the regeneration of cone pigments and of rhodopsin which had initially been bleached away to a small fraction of their dark-adapted levels. It is undoubtedly true that rhodopsin is substantially bleached at high adaptation levels and that it regenerates in the dark. Moreover at a moment when only 10 per cent of the full amount is present, it will need 10 times the intensity of test light to catch the same number of quanta. Here then is a necessary relation between dark adaptation and rhodopsin regeneration which has probably gone far to strengthen belief that the curves of Fig. 9 measure the regeneration of the visual pigments.

But this form of the photochemical theory is quite untenable. Consider the upper curve of Fig. 9. After 14 min of dark regeneration the threshold is still 2 log units above the final level. That means that during the first 14 min only 1 per cent of pigment has regenerated. Since, according to the photochemical theory, regeneration is fastest at the beginning and slows later, it would take not 40 min to regenerate completely but far longer than 1400 min. It therefore is not necessary to appeal for confirmation to the recent direct measurements on human rhodopsin during dark adaptation (see the next Section) in order to see that the form of photochemical theory just given can hardly be maintained. And indeed it does not appear often to have been explicitly advocated.

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But if not, what is the ground for supposing that the dark adaptation curve represents the regeneration of visual pigments at all? Presumably full recovery is delayed by the slowest mechanism in the complex chain which leads from the absorption of light to the appearance of nerve impulses and vision. But is it obvious that the slowest must be the regeneration of the pigment? And if not what are the grounds for supposing that in man rhodopsin takes half an hour to regenerate, and cone pigments 5 min? Though this opinion has been widely held for some 30 years no real support for it seems ever to have been advanced.

The only attempt to make a direct correlation between rhodopsin level and retinal sensitivity was that of GRANIT *et al.* (1939), who measured the E.R.G. in frogs and cats at various stages in dark adaptation. They also killed the animals at various stages and found, by direct extraction, how much rhodopsin had regenerated by that time. As they pointed out, the relation between sensitivity and rhodopsin level is not a simple one, and no E.R.G. is found at all until about half the rhodopsin has regenerated.

HECHT and many others have observed that curves such as those of Fig. 9 will correspond much better to the expected shape of the regeneration curve of a visual pigment if its concentration is represented not by sensitivity but by log sensitivity.

This involved the difficulty that though there is clear reason (given above) for accepting sensitivity there seemed none for accepting its logarithm. But recently WALD (1954) has advanced an important new concept from which the logarithmic relation necessarily follows. It is supposed that rods (and cones) contain their pigment molecules in compartments each of which will discharge in an all-or-none fashion upon absorbing its first quantum. Thereafter this compartment will absorb more quanta from time to time upon the remaining pigment molecules, and regeneration of the bleached molecules will also take place. But no further discharge will be possible from this compartment unless a quantum is absorbed after the full complement of molecules has been restored. By this scheme the threshold will be doubled, not when half the molecules have been bleached but when half the compartments have been inactivated—at the rate of about 1 molecule per compartment. The exact relation (WALD *et al.*, 1955) is given by the following simple formula

$$zN = 2{\cdot}3 \log I/I_0 \qquad \ldots.(3)$$

where I_0 = the absolute threshold,

I = the threshold when

z = fraction of molecules bleached, (assumed small),

N = number of molecules in a full compartment.

This theory certainly establishes a logarithmic relation between the threshold and the amount of rhodopsin bleached; what is not so clear is that this relation in fact exists. No full-scale experiments have ever been undertaken and the preliminary measurements by WALD (1954) are not very encouraging. He exposed the dark adapted eye to light for 5 sec, used the method devised by RUSHTON and COHEN (1954) for obtaining a *physical* estimate of the fraction of rhodopsin bleached, and measured the visual threshold immediately after extinction of the light. In three experiments using flashes which would bleach the rhodopsin by only 0·006, 0·19 and 0·59 per cent respectively, he found the instantaneous thresholds to be raised 8·5, 480 and 3300 times. When these values for z and I/I_0 are inserted in formula (3) above, the derived values for N in the three cases are 35,000, 3200 and 1400 instead of being constant. It might be thought that the difficulties in obtaining instantaneous threshold measurements could well account for the discrepancy between 3200 and 1400 in the value of N. But, as is seen from equation (3), to cover this divergence the threshold

would have to be in error by a factor not of 2 but of 600,000. In the opinion of the writer, WALD's hypothesis, in the very simple form given, is not quantitatively satisfactory. But the concept is a most stimulating one and likely to prove the starting point of important developments.

3.2. *Nerve changes in dark adaptation.* The determination of the "true" relation between threshold and rhodopsin level is much complicated by the fact that the fundamental assumption of the photochemical

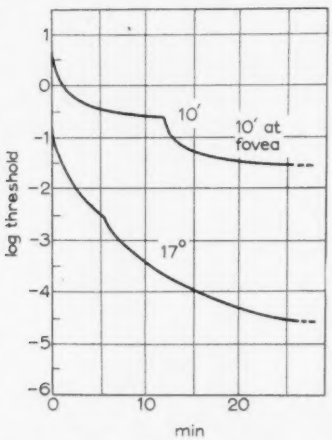

Fig. 10. Dark adaptation curves taken upon the same parafoveal region. Conditions identical except that test fields were in one case 17° and in the other 10′ (from CRAIK and VERNON, 1941).

theory—that the threshold depends simply upon the rhodopsin level is not correct. Fig. 10 shows two dark adaptation curves taken at the same retinal location (parafovea) in conditions identical except that the test stimulus was in one case 17° in size and in the other 10′ (CRAIK and VERNON, 1941).*

Now the bleaching and regeneration of rhodopsin must be the same in these two experiments since the only difference is the size of the test-field used to measure the degree of dark adaptation. Thus factors other than rhodopsin level have a profound effect upon threshold.

These factors clearly involve spatial interaction between receptors fairly widely separated upon the retina and it is hardly more than a restatement of the facts of Fig. 10 to say that as dark adaptation proceeds the nature of the spatial summation changes. Electrophysiological investigation fully supports this interpretation.

* The label "10′ at fovea" is misleading and has surprised the writer and many others. Prof. Vernon has explained that in the experiment the test light fell upon the parafovea (as is clear from the kinked curve). But it was judged to subtend 10′ by looking directly at it, and spherical aberration may have altered slightly this subtense in the experimental situation.

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In the first thorough investigation of optic nerve discharges ever undertaken, ADRIAN and MATTHEWS (1928) showed that the simultaneous illumination of 4 spots separated by as much as 1·2 mm on the retina of a conger eel (corresponding to an angular separation of 20° in water) reduced the latency of nerve discharge as compared with the illumination of one spot only; and that this spatial interaction was enhanced by strychnine. In an important recent paper BARLOW *et al.* (1957 a b) have analysed in some detail the nature of lateral interaction in the cat. Both KUFFLER (1953) in the cat and BARLOW (1953) in the frog had independently shown that in the light-adapted state the receptive field of a single ganglion cell consisted of a central region surrounded by an annular region of different character. Illumination of the centre gave a discharge of the ganglion either at "on" or at "off" depending upon the ganglion selected. Illumination of the annular region in addition always inhibited to some extent the discharge from the centre in the light-adapted state. In full dark adaptation this did not occur. From this previous work two questions naturally arose. In dark adaptation does the annular region change from inhibition to excitation so that it adds to the area over which summation occurs? And is the change associated with the Purkinje shift from cone to rod excitation?

The 1957 papers show that both these suggestions are wrong. An estimate of summation area may be obtained by observing how the threshold is lowered by increasing the size of the illuminated spot centred on the receptive field of the ganglion recorded. In the light adapted state the threshold is decreased as the spot is enlarged up to a diameter of 1 mm (on the retina) and then the threshold rises again as the inhibitory annulus is also excited. In dark adaptation there is the same fall of threshold up to 1 mm diameter but no further change when light falls upon the annulus. Thus the inhibition in this region is cut off, but not converted into excitation.

This cut-off of *lateral inhibition* occurred rather abruptly near the end of dark adaptation, usually long after the cut-off of cones associated with the Purkinje shift. Moreover, at moderate light adaptations, the rods and cones in the annulus were found to contribute to inhibition in about the proportion that they contributed to excitation at the centre.

Thus it is plain that at various levels of adaptation the threshold depends to a marked degree upon the synaptic organization of the retina and in particular upon the amount of lateral inhibition, so it would be rather surprising if a photochemical theory which failed to take such matters into account could predict thresholds at all accurately.

3.3. *Rod discrimination.* HECHT's real success with the photochemical

theory was his explanation of how the Weber fraction $\Delta I/I$ depended upon the adaptation level I. He calculated the equilibrium level of rhodopsin under steady illumination I, and assumed that the superposed flash ΔI would just be seen when it decomposed "a constant increment of sensitive material" (HECHT, 1937, p. 275). No more reasonable assumption could be made, and the fit is excellent both with his own measurements and with those of former workers. Fig. 11 shows the increment threshold measurements of AUBERT (1865) as replotted by HECHT (1935) together with his theoretical curves (each branch of

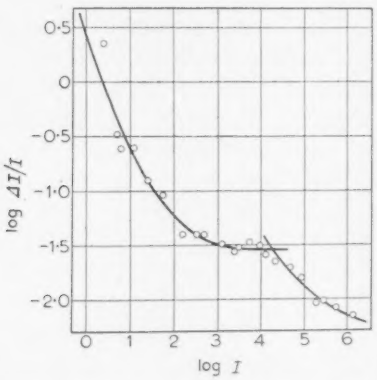

Fig. 11. AUBERT's measurements of intensity discrimination, with HECHT's theoretical curves. Log I should be diminished by about 4 to convert the rod branch to log trolands (from HECHT, 1935).

which may be moved without rotation to get the best fit). The left branch represents the rods, and the curve is that derived from the assumption that photolysis is mono-molecular and regeneration bi-molecular.

The success of this theoretical treatment was extended to other visual relations and as may be seen in HECHT's (1937) review an impressive body of experimental fact fits excellently the curves derived from the foregoing theory. The observations relate I, the level of adaptation, with acuity, the fusion frequency of flicker, and the instantaneous threshold when I is extinguished, in addition to the increment threshold (Fig. 11). This extended achievement is more a tribute to HECHT's enterprise and resource than to the soundness of his theory. For having shown for rods that all these experimental functions suitably plotted coincided, it must follow that any theoretical curve which fitted one would fit all and the only thing left was to explain why the chemical process represented by the theoretical curve corresponded with the limiting sensation of flicker or whatever was the sensory correlate.

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However, the real success of HECHT's treatment was immense and the impact of his ideas so considerable that results of psychophysical experiments to-day are still being interpreted in terms of the equilibrium levels of rhodopsin and cone pigments as he postulated them. It is therefore worth-while to draw attention to three things which detract from the satisfactoriness of this aspect of the photochemical theory of visual function.

(i) HECHT supposed that a flash is just seen when it decomposes a constant increment of sensitive material. If this is valid while I is shining (Fig. 11) and also for the first moments of dark adaptation (instantaneous thresholds), we should expect it to hold for all subsequent times during dark adaptation. But this is precisely the form of the photochemical theory which had to be rejected earlier as quite untenable since, in relation to the upper curve of Fig. 9, it requires that only 0·1 per cent of rhodopsin is regenerated in the first 12 min of dark adaptation.

(ii) The way in which synaptic interaction depends upon the level of adaptation is bound to affect increment thresholds very much as we have seen it does dark adaptation thresholds. Its importance was stressed by LYTHGOE (1940) who was himself an outstanding contributor to the chemistry of visual pigments, and the recent paper of BARLOW (1957) shows that the curve of log $\Delta I/I$ plotted against log I has quite a different shape when the test flash is small and brief on the one hand, or large and long on the other. No curve which fits the first can be shifted parallel to itself to fit the second, even approximately. It should be stated that HECHT always insisted that his theory was a gross over-simplification of the full facts, nevertheless he presented it as essentially correct for the isolated relations studied.

(iii) We must always be careful with our arbitrary constants. They have a demi-mondaine status, and though we cannot all do without them, the sooner they are respectably married to physical measurement the better. What is the actual value of log I in Fig. 11 at which rhodopsin is believed to be half bleached? It lies between the numbers 2 and 3 which corresponds to about 0·03 trolands of retinal illumination. But it is easy to calculate that 0·03 trolands amounts to the incidence upon the retina of about 10^7 quanta/cm^2 sec, or 1 quantum per rod in 3 sec. It would take a long time to bleach half the rhodopsin away with this light even if no regeneration occurred. DARTNALL *et al.* (1938) found that it needed about 10^{16} quanta/cm^2 to fall upon rhodopsin in order to bleach half away. So at the rate of 10^7/cm^2 sec it would take 30 years.

The realization that in steady scotopic conditions rhodopsin is virtually unbleached has brought about the collapse of the photochemical theory of scotopic function. HECHT himself was a pioneer in

the new quantum approach (HECHT *et al.*, 1942) but it was BAUMGARDT (1950) who first made it plain that in the scotopic state we are concerned, not with chemical concentrations reacting by chance encounter, but with an organization of single molecular triggers integrated by nerves.

4. *The measurement of rhodopsin in human rods*

4.1. *Principles of measurement.* As is clear from the foregoing Section, one of the chief difficulties in assessing the photochemical

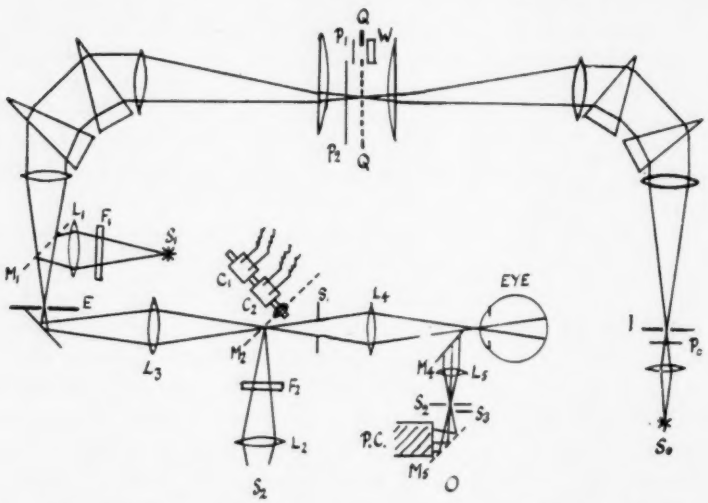

Fig. 12. Reflexion densitometer for measuring human visual pigments in normal and colour blind (from RUSHTON, 1957c).

theory was in knowing what *was* the level of rhodopsin in equilibrium at a given brightness of illumination. Recently CAMPBELL and I have succeeded in measuring this physically in normal human eyes (RUSHTON *et al.*, 1955; CAMPBELL and RUSHTON, 1955; RUSHTON, 1956 a b, 1957b).

It is well known that the back of the eye, the *fundus*, may be observed in an ophthalmoscope. A diagram of this arrangement is seen in Fig. 12, where light from L_4 enters the eye through the upper half of the dilated pupil (homatropin) and illuminates a patch of retina. Some light scattered from the back of the eye leaves through the lower half of the pupil and falling upon the mirror M_4 is deflected downwards. An observer's eye placed at L_5 will see an image of the *fundus*, formed by light which has *passed twice through the retina*. If the incident light was green, the rods if they could individually be seen would doubtless appear somewhat dark in the dark adapted state and transparent when bleached as in Plate I (*a*), I (*c*) (from the frog). Though the rods themselves are not seen, the amount of light reflected after a double

passage through the retina may be measured by a photocell, and in particular the increase found after bleaching. If now a neutral density is introduced into the path of the incident light of such a value that the increased reflected light is just returned to the value before bleaching, it is plain that the density so interposed must be equal to the (double) density of pigment removed on bleaching. In this way the pigment density may be measured objectively simply by the adjustment of an interposed photometric wedge. Several forms of ophthalmoscopic densitometer have been built differing considerably in lay-out. In all

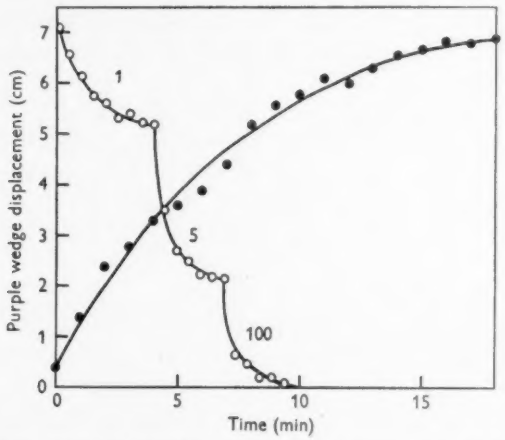

Fig. 13. Circles, effect of bleaching rhodopsin in human eye by lights of 1, 5 and 100 units of intensity, (1 = 20,000 trolands); dots, regeneration of rhodopsin in the dark (from CAMPBELL and RUSHTON, 1955).

of them the light fraction which is partly absorbed by the visual pigment is flickered against a light fraction from the same source which cannot be absorbed and thus serves as a check upon fluctuations of light source, gain of recording equipment, etc. Fig. 12 shows the most versatile form of the instrument, built round STILES' (1955) double monochromator. The wave-lengths used are selected at the spectrum QQ and focused upon the cornea of the subject. The light reflected from the *fundus* is brought to a focus at S_2 which is a stop admitting light only from the 3–5° area of retina measured. This light is received upon a photo-multiplier tube P.C.; S_1 and S_2 are additional light sources for bleaching.

4.2. *Results.* Fig. 13 shows the effect of exposing the left eye of the writer to three intensities of light in succession: 20,000 trolands, 100,000 and finally 2,000,000 trolands (circles). The dots show the effect of remaining a further 18 min in the dark. The ordinates represent the shift of a photometric wedge (purple in this case) which

replaced the visual purple removed by bleaching. The claim is, therefore, that the circles represent the bleaching of rhodopsin and the dots its regeneration in the dark.

Now the intensity of light reflected from the eyes depends upon so many factors besides the density of visual pigments that despite all care in the design of equipment one needs to make a strong case that change in position of the photometric wedge really does signify a corresponding change in pigment density. Support for this view is derived from the following measurements.

(i) When the 3° region of the retina bleached did not coincide with the 3° region measured but lay just beside it, no change in wedge setting followed the strongest bleaching. Thus the wedge measures a *local* effect of bleaching.

(ii) The result of a brief flash was instantly to cause a new position of balance for the wedge. Starting at the dark adapted state, the new position was found to be the same for different intensities of flash I and different times of exposure t provided $I \times t$ was constant and t less than 40 sec. Thus whatever is measured obeys the Bunsen–Roscoe law up to 40 sec.

(iii) The flash duration was now kept constant at 30 sec but the colour of the light used varied throughout the spectrum, the brightness being always such that when attenuated 10,000 times it matched a fixed faint field by twilight vision. These coloured lights were thus adjusted in brightness to bleach rhodopsin equally. They were found in fact all to produce the same wedge shift, and this was not due to insensitiveness to detect a change. For, when the bleaching light was altered slightly in brightness from the matched value, a change in wedge shift was observed.

(iv) The amount of wedge shift produced by a 10 sec bleach using lights of various intensities, corresponds to the calculated curve to be expected of a visual pigment. The log number of incident quanta/cm^2 required to bleach half this pigment away was 15·72, that for rhodopsin being 15·94 (see p. 263). Thus the wedge displacements are directly proportional to the density of a visual pigment whose photosensitivity lies close to that of rhodopsin.

(v) The difference spectrum is similar to that of rhodopsin but shifted somewhat into the red as would be expected since the orange photoproducts do not have time to decompose entirely.

(vi) An attempt was made to estimate the amount of rhodopsin in various regions of the retina by finding the amount of change produced by a total bleach. The points in Fig. 14 show the wedge change (by two different techniques) and the curve indicates the number of rods/mm^2 (Østerberg, 1935). This also supports the belief that rhodopsin is what is measured, for there is none of it on fovea or

optic disc and elsewhere it goes roughly hand in hand with the rod density. But, although there is this general agreement, it is clear that the rhodopsin value rises and falls more than in proportion to the rod concentration. This, however is just what would be expected if where the rods are less tightly packed more light is returned from the (brighter) spaces between them. And indeed, a further analysis of this very feature leads to an estimate of the actual density of rhodopsin in the living human rod.

If now it is accepted that rhodopsin is measured by this technique two results become available. One is the maximum density in the rods, the other the proportion bleached at various stages in light-dark manoeuvres. The full density in a rod (for single light passage) lies

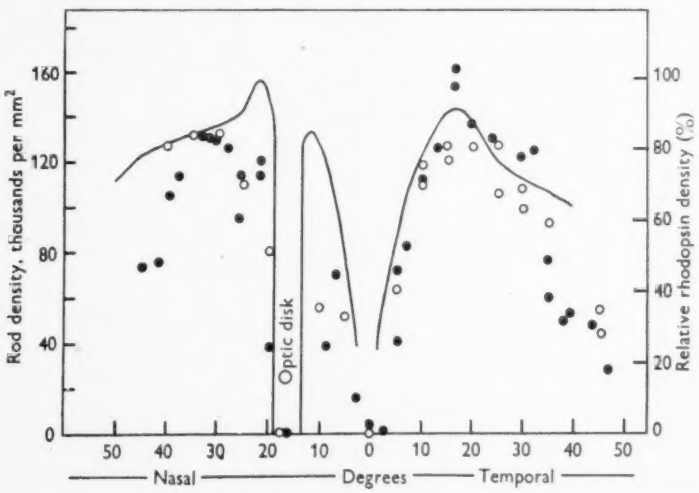

Fig. 14. The curve shows the rod density as a function of the angular distance from the fovea along the horizontal meridian (OSTERBERG). Circles and dots, rhodopsin density as measured by retinal densitometry (two methods) (from CAMPBELL and RUSHTON, 1955).

between 0·1 and 0·2, 0·15 being a reasonable estimate (RUSHTON, 1956b). This means that rhodopsin will absorb 30 per cent of the green light which falls upon a rod, 20 per cent of that which falls upon the retina and 10 per cent of that which reaches the cornea.

Fig. 13 gives some indication of rhodopsin equilibrium in bleaching lights of various intensities. Half-bleach will correspond to about 40,000 trolands which is just a million times the intensity postulated by the theoretical curve of Fig. 11.

Regeneration in the dark is given by the dots of Fig. 13 which do not actually level off until some 30 min have elapsed (see RUSHTON *et*

al., 1955). Thus the belief that the regeneration of rhodopsin takes half an hour to be completed turns out to be correct. This, moreover, is no coincidence, for in the cat both dark adaptation and rhodopsin regeneration are 3–4 times as slow as in man (GRANIT *et al.*, 1939; BARLOW *et al.*, 1957a; WEALE, 1953) (the same has just been demonstrated by comparing frogs with alligators (WALD *et al.*, 1957)), and, as will appear later, cone pigments regenerate in about 7 min from a strong bleach such as used for the upper curve of Fig. 9.

These direct measurements of pigment bleaching and regeneration therefore lead to the conclusion that the return of excitability waits upon the return of a full complement of regenerated molecules rather than the independent restoration of other parts of the mechanism of visual excitation. And just as a flash which bleaches only 1 per cent of the rhodopsin will raise the threshold many times, so the last 1 per cent of rhodopsin to be restored appears to be associated with an appreciable fall in the threshold of dark adaptation.

These results certainly encourage a modified form of WALD's compartment theory, but we need to know what is the experimental relation between rise of threshold and pigment bleached, how constant is this relation under various conditions of bleaching and regeneration, and how far must we allow for changes in lateral inhibition and in summation time in our interpretation of threshold values.

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IV. VISUAL PIGMENTS IN THE CONES

1. *Spectral sensitivity*

In man cone vision is associated with bright surroundings, good acuity, short latency of response and the appreciation of colour, and this pattern seems to underlie cone function in vertebrates generally. There is wide agreement that strong light signals allow, and photopic mechanisms secure a more exact appreciation of the retinal image in space and time. There is less accord upon the subject of colour vision in animals.

One reason for this is the use of "colour vision" in several distinct senses. Two colour relations which concern the retinal physiologist are the spectral sensitivities of the various photo-receptors and that of the various ganglion cells in specified conditions. Moreover as techniques advance (MACNICHOL *et al.*, 1957) the spectral sensitivities of other elements in the chains of neurones become available and important. At higher levels of integration, the reported sensation in man and the observed behaviour in trained animals may distinguish those colour combinations which are confused from those which are not. But with animals, it is not always clear how to interpret negative results. There is a great difference between concluding "this experiment shows that

cats cannot tell red from green" and "this experiment does not show that cats can tell red from green." It is important also to appreciate the difference between detecting some sensory quality and remembering it. Many people who can easily say which is the upper of two notes played in succession upon the piano will not be able to recognize it as the upper one when played by itself next day.

No pigment has ever been seen in a cone and the only evidence we have about cone pigments is derived either from extracts of cone-rich eyes, measurements by ophthalmoscopic densitometry upon the human fovea, or inference from physiological responses to coloured lights. As mentioned earlier (II, 2.2) an extract of fowls' retinas gave in addition to rhodopsin a second pigment iodopsin whose maximum absorption lay at about 560 mμ and which would regenerate from 11-*cis* retinene some 500 times as fast as rhodopsin (WALD *et al.*, 1955). The fowl's retina contains chiefly cones and hence it is natural to conclude that iodopsin is the cone pigment, especially as the absorption spectrum is not far from the photopic (= daylight) sensitivity curve of the fowl (HONIGMANN, 1921) or pigeon (GRANIT, 1942b; DONNER, 1953). The densitometry of the human fovea will be discussed in the next Section and we turn now to physiological responses which might throw light upon the spectral sensitivity of cones.

The microelectrode technique of GRANIT which showed that scotopic sensitivity coincided accurately with the absorption of rhodopsin reveals in most animals a curve—"the photopic dominator"—which lies close to the absorption of iodopsin. One might expect that the correspondence would be closest in the fowl from which this pigment was in fact extracted, but the cones of hens and pigeons contain oil droplets of various colours which since SCHULTZE (1866) have been regarded as of importance to their colour vision. Since these droplets make one cone differ from another in spectral properties and none will have the spectral sensitivity of iodopsin, it would be surprising if any response from such a retina corresponded closely with iodopsin absorption. But mammals and frogs have no coloured drops and, as may be seen in Fig. 15, the photopic dominator sensitivity of such animals fits iodopsin nearly as well as scotopic sensitivity fits rhodopsin. But this does not make the iodopsin position nearly as secure as the rhodopsin position.

Scotopic vision is monochromatic, all colours are equivalent when intensities are scaled inversely as rhodopsin absorption, and the rods can be seen all to contain only one kind of pigment (in the mammal). None of these statements is known to be true for cone vision and most are demonstrably false. Some ganglion cells (modulators) exhibit a sensitivity curve which is quite different from iodopsin both because the maximum does not lie near 560 mμ and because in shape the curve

is much narrower (GRANIT, 1942a). The presence of modulators in addition to the iodopsin-like dominators in the same preparation has received two types of interpretation which may be stated over-crisply as follows. *Either* each ganglion-cell is connected to one type of photoreceptor, so that dominator receptors contain iodopsin and each type of modulator receptor contains the appropriate narrow-band pigment. *Or* most ganglions are connected to most types of receptor in various ways; dominator ganglia add the receptor sensitivities: modulator ganglia subtract them. An objection to the first suggestion is that no narrow band pigments have ever been extracted and there is no good

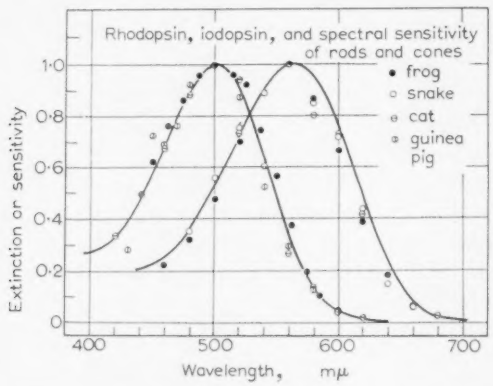

Fig. 15. The curves show the extinction spectra of fowls' rhodopsin and iodopsin. The points plot GRANIT's scotopic and photopic dominator curves for the animals indicated (from WALD *et al.*, 1955).

evidence that they are involved in human colour vision. An objection to the second is that if the dominator is formed by nerve integration it is surprisingly stable under differential adaptation, and the correspondence with iodopsin (Fig. 15) is a striking coincidence.

It might be thought that one could tell whether the dominator ganglion was connected to one or to more than one kind of receptor by suddenly substituting one colour for another. If only one kind of receptor was involved, any two colours suitably scaled in intensity would be equivalent for the receptor, and thus the change would not be detected. With two kinds of receptor connected to the ganglion at least one of them would detect the change whatever the scaling. It may be recollected that the spectral sensitivity curve of Fig. 8 was analysed by the Stiles–Crawford retinal directional effect, and the region to the right of 500 mμ was shown to involve cones while that to the left involved rods only. Now it was found (DONNER and RUSHTON, 1959a) that when the eye was adapted to a light represented by any point on the curve, this light could be suddenly changed to any other

light on the curve without the substitution being detected by the ganglion cell. This was not due to general insensitivity, for the substituted light had only to be a little too weak or a little too strong for a ganglion discharge to signal the change-over. The rod-cone combination upon this ganglion behaved to substitution exactly as would a single kind of receptor whose pigment absorbed like the dotted curve of Fig. 8. This does not encourage us to argue with much confidence from ganglion responses back to the supposed visual pigments in the receptors.

However, the analysis of human cone vision by means of increment thresholds has proved outstandingly successful in the hands of STILES (1949, 1953), and there is good reason to hope that its application by DONNER (1959) to the ganglion cell responses in the frog will prove correspondingly fruitful.

2. *Foveal pigments in man*

2.1. *Reflexion densitometry.* In the measurement of rhodopsin upon the human retina by the method of reflexion densitometry it was found

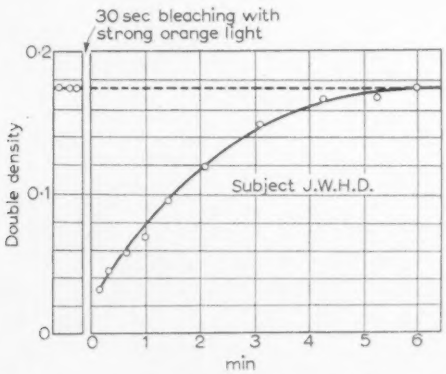

Fig. 16. Bleaching and regeneration of foveal (cone) pigment in man. Ordinates give double density change of pigment measured in yellow light (580 mμ). Strong orange light bleaches, and regeneration occurs in the subsequent 6 min in the dark (from RUSHTON, 1957a).

(Fig. 14) that there was no rhodopsin upon the fovea. This was to be expected, but it might be thought that the foveal cone pigments would have produced some change in reflected light upon bleaching. The measurements of Fig. 14 were made, however, in conditions sensitive to detect rhodopsin but insensitive for cone pigments. With modified conditions (RUSHTON, 1957a) a substantial density change can be measured upon the human fovea as shown in Fig. 16. The change is due to a photosensitive pigment since the amount bleached with

flashes of different brightness fits the theoretical curve for such a chemical. But the pigment is not rhodopsin for that would be neither bleachable by the orange light used nor measureable by the yellow. Moreover, as seen in Fig. 13 rhodopsin takes half an hour to regenerate whereas the foveal pigment takes 7 min (cf. rod and cone portions of the dark adaptation curve Fig. 9).

The relation of cone pigment to cone vision is seen most easily in *protanopes*. Nearly all colour blind people have difficulty in distinguishing red from green. A protanope is an extreme form where

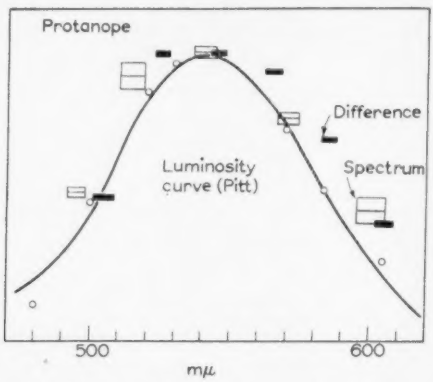

Fig. 17. Difference spectrum of foveal pigments in the protanope (black and white rectangles). Curve, protanope luminosity (PITT); circles, confirmation of this on one of the subjects investigated (from RUSHTON, 1957c).

green is quite indistinguishable from red (with a small amount of blue added) and where the matched red appears very much brighter than the green as judged by normal vision. Fig. 17 shows the difference spectrum of the foveal pigment of the protanope (RUSHTON, 1957c). It was found to be the same after partial bleaching by orange or green lights indicating that only one pigment is present. This is the condition where we can predict that the absorption spectrum should correspond with the subject's spectral sensitivity. The curve of Fig. 17 is the protanope sensitivity given by PITT (1944) who found it very constant from subject to subject. The protanope therefore has monochromatic vision in the green–red range because he has only one visual pigment—a green sensitive one.

It is natural to wonder why there is no mention of a blue sensitive pigment seeing that a protanope has blue sensitivity. No measurements have been successful with *any* subject in exhibiting a blue-sensitive foveal pigment. This may be because the blue receptors which contribute so little to luminosity also contribute little to total light absorption; or the "blue blindness" of the central fovea (KÖNIG, 1894; WILLMER

and WRIGHT, 1945), may be due to scarcity of foveal receptors for blue; in any case the yellow macular pigment seems to diminish the "blue" signal so much that reliable measurements at short wave-lengths are difficult.

The difference spectrum determined by partial bleaching upon the *normal* fovea reveals two visual pigments as indicated in Fig. 18. One of these is the same as that of the protanope, as was to be expected since the protanope accepts normal colour matches which must almost certainly mean that such pigments as he has are normal ones. The

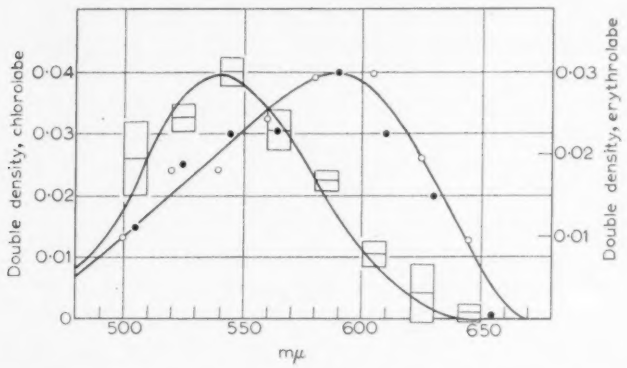

Fig. 18. Difference spectrum of two pigments, upon the normal fovea. The left curve is PITT's protanope sensitivity. The other is drawn freehand through the points (from RUSHTON, 1957c).

other pigment in the normal eye absorbs maximally at about 590 mμ. It is the absence of this which makes the protanope "red blind."

It is convenient to have names for the three visual pigments of normal vision which absorb red, green and blue light. They may be called *erythrolabe*, *chlorolabe* and *cyanolabe* respectively.*

Difference spectra are notoriously untrustworthy even when the conditions of measurement are far more satisfactory than the reflexion from the black back of the human eye, and so far there is no secure human sensitivity curve to support the difference spectrum of erythrolabe as the protanope supports chlorolabe. It might be hoped that such

* Since pigments are studied by their absorption spectra and receptors by their sensitivity, there is an element of confusion in naming the pigment involved—e.g. visual purple, rhodopsin—by the colour which is *not* absorbed and to which the receptor is insensitive. To go further and name the invisible pigment of the human cone by the colour it would exhibit if it could be accumulated sufficiently to be seen savours of perversity. The literature of human colour vision abounds with references to "red, green and blue receptors," in which the red receptor is not supposed to have a ruddy appearance but to *absorb* red light relatively well. *Erythrolabe* (= red-taking) follows in this tradition.

support could be derived from the sensitivity of deuteranopes—the other type of red–green dichromat, sometimes called "green blind." But though the deuteranope has monochromatic vision throughout the red–green region of the spectrum, partial bleaching shows clearly that his fovea contains *both* the normal pigments (with rather more erythrolabe and less chlorolabe than normal). What he lacks then is not the pigments but their proper employment.

The densities given in Fig. 18 are from partial bleaching only. Total densities for double transit are for chlorolabe about 0·06 and for erythrolabe 0·12. This is a density comparable with that of rhodopsin in the human rods (Rushton, 1956b), so it might be wondered why cone pigments are so much harder to see or to extract. It seems likely that though the density is high, the *total quantity* is very small. If in the cones both light and pigment were concentrated at some "point" we should expect high density with small quantity, the Stiles–Crawford effect (cones only) and the great cone photosensitivity found by Brindley (1955) and Rushton (1957a, c). The pigment would not easily be seen except when viewed from the vitreal aspect (as with the ophthalmoscope) but in this case the "point" of pigment would be magnified to fill the whole cone and so perhaps explain the surprising fact that human cone pigments can be measured at all.

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2.2. *Breakdown of colour matches in adaptation.* Since Von Kries (1878) it has been well known that adaptation to a coloured light of moderate intensity will cause spectral colours to appear changed in hue, but that any two colours which matched before adaptation continue to appear a good match afterwards. If, however, the adapting light is of high intensity, colour matches are found to break down (Wright, 1936). It has sometimes been claimed that the upset of matches could be due to alteration in nerve pathways even if the spectral absorption curves of the visual pigments remained unchanged after adaptation. We have seen earlier that thresholds can be greatly affected by variation in nerve pathway, but this cannot alter a colour match (see Brindley, 1957 for a more rigid presentation of the following argument).

For the condition that

$$\text{red} + \text{green} = \text{yellow} (+ \text{blue})$$

for instance requires that *each* of the three visual pigments should absorb quanta at the same rate from both of the matched fields. And if, after adaptation, the absorption spectrum of each pigment remains unchanged in shape, then each pigment will still receive quanta equally from the two fields and hence there will be no photochemical difference to form a possible basis of discrimination between the two fields. It follows that the breakdown in colour matches which is found after

adaptation to very bright lights must involve a change in the absorption spectrum of at least one visual pigment. The nature of this change has been strikingly analysed by BRINDLEY (1953, 1955).

In the first paper he measured the intensity of a red light (680 mμ) and of a green light (550) which when mixed matched a fixed yellow (578). He found that for all intensities and colours of very intense adaptation the change in the logarithms of red and green light energies required for the match were always related by

$$\Delta \log R = -5 \Delta \log G \qquad \ldots.(4)$$

This is exactly what would be expected if some alteration occurred in the red pigment erythrolabe and in that only.

Now the simplest change which can occur in the absorption spectrum of a pigment is the removal of self-screening as the high initial density is reduced on bleaching. The relation is shown in Fig. 7 where the dotted curve changes to the continuous curve as the density falls from 0·7 to 0·2. Could an initial high density of erythrolabe cause the alteration of the red–green match simply by the removal of self-screening?

Several lines of evidence point to this explanation of which two may here be mentioned. If the eye is adapted to an extremely bright blue-green light, then for a period of 10–15 sec the whole spectrum from 500 to 700 mμ may be matched with yellow. The quantum energies at different wavelengths for this match should thus give the absorption spectrum of erythrolabe in low concentration, for such strong adapting lights even at 497 mμ are found to bleach it substantially. Now this low-concentration absorption spectrum turns out to be related to the normal absorption spectrum as are the two curves of Fig. 7.

It might happen that light falling obliquely upon the cones (by entering through the periphery of the dilated pupil) would not traverse so great a depth of pigment as light falling normally. If this were so, colour matches would break down upon decentring the light through the pupil in much the same way as they do upon strong adaptation. In fact STILES (1937) found that monochromatic lights do appear to change hue upon decentring and BRINDLEY (1953) showed that colour matches break down in the expected way. His explanation was clinched when BRINDLEY showed that decentring had no effect upon colour matches when a strong adaptation had abolished the self-screening upon which the explanation hangs.

But if the break-down of a colour match is the result of the partial or complete removal of the self-screening in erythrolabe, it may be used to measure the density of this pigment in various conditions. This is the analysis in BRINDLEY's second paper (1955) the results of which were as follows. The normal density of erythrolabe was 0·98 (single light passage), the regeneration of the pigment was complete by

about 6 min, and its photosensitivity was about 18 times that of rhodopsin extract.

Direct measurements by reflexion densitometry has confirmed much of this, but no efforts to detect self-screening have been successful. The total density of erythrolabe may well be 0·98 within the depths of the cone, but if so the light which emerges and is measured in the photocell must have been reflected from the shallows.

2.3. *Kinetics of bleaching and regeneration.* We may avoid the difficulties of handling the kinetics of two pigments simultaneously by bleaching with white light. This is found to affect erythrolabe and chlorolabe equally, and since they also regenerate at equal rates in the dark, the two pigments throughout may be treated as one. The experiment consists first in bleaching for 10 sec with a strong light I_1 and measuring the fraction x of pigment which is bleached away. Then the bleaching light is reduced to a value I_2 such that the pigment is neither bleached further nor allowed to regenerate, but stays indefinitely in equilibrium (RUSHTON, 1958).

Now during the 10 sec exposure to I_1 there is no time for appreciable regeneration and the Bunsen–Roscow law is found to hold. Thus we may write

$$\frac{dx}{dt} = k\,(1 - x)I$$

$$\therefore \qquad -\log_e (1 - x) = kIt_0$$

where k is a constant and t_0 = bleaching time = 10 sec.

Now when I_1 has a certain value I_e (which turns out to be 5×10^5 trolands), $1 - x$ becomes 1/e at the end of the 10 sec bleach.

$$\therefore \qquad 1 = kI_e \,.\, 10$$

$$\therefore \qquad \frac{dx}{dt} = \frac{1 - x}{10} \cdot \frac{I}{I_e} = \frac{(1 - x)I}{5 \times 10^6} \qquad \ldots\ldots(5)$$

From this expression we know the *absolute* value of the bleaching rate at any level x under any intensity I. In particular we know it for each value of I_2 which maintains x in equilibrium. But in equilibrium the bleaching rate must be exactly equal to the regeneration rate, hence from (5) we may measure the rate of regeneration at various levels of x *while bleaching* was going on. Fig. 19 (circles) shows the rate plotted against x and it is seen that the results fall about a straight line through the origin, so that for any fraction x bleached the rate of regeneration is $x/130$ per sec.

But the rate of regeneration *in the dark* at each level x may be found by drawing the tangent to the curve of Fig. 16. These values are

plotted on Fig. 19 as dots, which are seen to fall within experimental limits upon the same line. Thus it appears that regeneration is a process dependent upon x but not upon I, so it may be simply added to the bleaching kinetics (5) at every stage. The full kinetics may therefore be written

$$\frac{dx}{dt} = \frac{(1-x)I}{5 \times 10^6} - \frac{x}{130} \qquad \ldots.(6)$$

where t is in seconds, I in trolands and I is any known function of t.

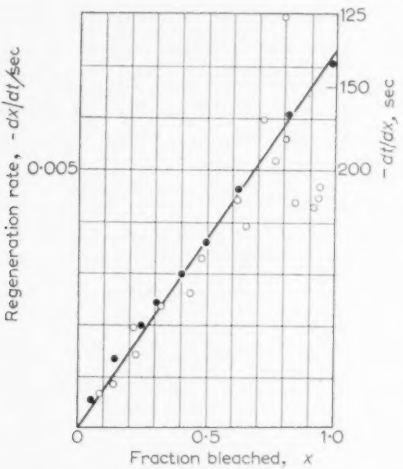

Fig. 19. The regeneration rate of human foveal pigments for various fractions x of pigment bleached. Dots, regeneration rate during dark adaptation; circles during equilibrium bleaching at lights of various intensities (from RUSHTON, 1958).

In the steady state we obtain one form of HECHT's celebrated equation

$$I = \frac{x}{1-x} \cdot 4 \times 10^4 \qquad \ldots.(7)$$

but here there are no arbitrary constants.

The light intensity postulated by HECHT for half-bleaching was some 30 trolands; but from (7) we see that the actual value is about 1000 times as great.

Now the kinetics of the regeneration of visual pigments have been studied by WALD and his colleagues who showed that a bimolecular reaction was involved, e.g.

$$\text{retinene}_1 + \text{photopsin} \rightarrow \text{iodopsin} \qquad \ldots.(8)$$

In their experiment a fixed amount of 11-*cis* retinene was suddenly added to the opsin, but this is not the condition in the eye where

retinene is in equilibrium with vitamin A and nearly all in the reduced form.

$$\text{Vitamin A} \underset{\text{DPN}}{\overset{\text{DPN}-\text{H}_2}{\rightleftharpoons}} \text{retinene} \qquad \ldots.(9)$$

The result of (9) is that the retinene concentration in the retina will remain at a very low but practically constant level, and hence from (8), the rate of iodopsin synthesis will be simply proportional to x the concentration of free photopsin. This is precisely the relationship shown in Fig. 19. Thus though *in vitro* regeneration is bimolecular, in the kinetics of the retina, the minute but constant level of retinene makes it in effect mono-molecular.

2.4. *Application to human colour vision.* In the previous volume of *Progress in Biophysics*, BRINDLEY (1958) has covered the field of human colour vision using evidence derived mainly from experiments in which two coloured lights are or are not just detected as distinct in the judgement of a normal observer. As BRINDLEY points out, this is a less objective study of phenomena than is used in the rest of physiology—or indeed in science generally. But some quite objective criteria may be applied to human vision if the effectiveness of a coloured light is judged, not by the sensation produced, but by the E.R.G. or by the effect in bleaching visual pigments measured by reflexion densitometry.

The inaccuracies in measuring foveal pigments in this way are still too serious to allow much more to be said than the contents of BRINDLEY's half page on the subject. The fact that the protanope lacks the red-sensitive pigment erytholabe, is strong evidence that protanopia is a defect primarily in the pigment, rather than in any other part of the visual mechanism of retina or brain. And the fact that the difference spectrum of protanopes fits closely their visibility curve suggests that the difference spectrum, as measured, cannot be very far from representing the action spectrum of chlorolabe.

But we are upon far weaker ground with erythrolabe. There is an uncertainty of about 0·01 in the (double) density measured in a single experiment. This is not serious in the difference spectrum of chlorolabe in the protanope where the total double density may be nearly 0·2. But erythrolabe in normal eyes (Fig. 17) has only been measured alone by a partial bleach using a deep red light which does not affect chlorolabe at all. As is seen (Fig. 17) the double density change in this partial bleach is only about 0·03 and hence little confidence can be placed upon the exact shape of the curve so determined.

Moreover the work of BRINDLEY upon the breakdown of colour matches after adaptation to very bright lights points strongly to erythrolabe being present with a density sufficient to give self-screening.

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But no self-screening has been detected by reflexion densitometry, so it is probable that no appreciable fraction of the light which goes deep enough into the cones to excite vision ever returns to excite the photocell. In that case the action spectrum of erythrolabe, however accurately determined, would correspond not to the "red sensation" but rather to that reduced form of it which results from adapting to very bright lights.

Enough has been said to indicate that at present reflexion densitometry has little to contribute to colour vision. The measurements support KÖNIG (1903) in accepting protanope visibility as the green sensation. There is no contribution to the blue sensation whatever, and the difficulties which have always centred round the red sensation are not in any way cleared up.

But measurements upon human pigments have only just begun, and it is to be hoped that far better experiments will be made. When they are, it is likely that they will define the coefficients for transforming the well known colour mixture curves into the absorption spectra of the three visual pigments. In this way the imperfections of reflexion densitometry will be supported by the accurate colour matches which have already been made by WRIGHT (1946) and by STILES (1955).

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MECHANICAL INTO ELECTRICAL ENERGY IN CERTAIN MECHANORECEPTORS

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MECHANICAL INTO ELECTRICAL ENERGY IN CERTAIN MECHANORECEPTORS

J. A. B. Gray

I. Introduction

The success, in a biological sense, of an animal depends to a great extent on its ability to maintain its internal environment constant and to respond appropriately to external events. To achieve these ends, control mechanisms have developed, and in many species these have grown into highly complicated nervous systems. The ability of any nervous system to provide an adequate degree of control depends, amongst other things, on the adequacy and accuracy of the information it receives. This information about the state of the internal environment and the events of the external world is provided through the activity of a large number of receptors. There are many types of receptor and it is one of their important properties that each type is specifically sensitive to one or more forms of energy; normally a receptor is much more sensitive to one form than to others; thus there are receptors, the mechanoreceptors, that are particularly sensitive to various functions of mechanical energy and others, the photoreceptors, thermal receptors and chemoreceptors that respond to electro-magnetic (light), thermal and chemical energy respectively. Sometimes receptors are grouped into complex organs, such as the eyes and ears of vertebrates, but many work individually and are scattered throughout the skin and other tissues. It is, in many ways, easier to analyse the mechanism of these isolated receptors and evidence from isolated mechanoreceptors will form the substance of this article.

1. *Signalling of information*

Information is transmitted from the receptors to the central nervous system along the afferent nerve fibres by the passage of nerve impulses. These impulses are, in any one fibre, always identical and the only variable quantity in any one unit is the interval between the impulses. Exactly what this quantity signals depends on the nature of the unit; thus there are receptors in muscles that signal length and others in vessel walls that signal the pressure in the vessel. In these instances a particular length or pressure is related to a particular frequency of nerve impulses, and this relationship is, except during or soon after a rapid change in the stimulus, independent of time. The response of

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any one unit will vary with, say, the pressure over a certain limited range, the frequencies changing from zero up to values which may reach several hundreds per second; different units often have different operating ranges. For all types of unit, however, time is a very important factor; those that signal steady states, such as the examples just given, also give responses that are related to the rate of change of state and there are many other types of receptor that respond only to changes of state.

2. *Initiation of nerve impulses*

Nerve impulses are initiated by receptors, but before considering how this happens it is necessary to consider the initiation of nerve impulses in general terms. A nerve fibre is essentially a tubular structure filled with a solution of electrolytes and bounded by a membrane, which has a relatively high electrical impedance and which has certain special properties related to its permeability to ions. The fibre lies in a medium which has an electrolyte composition different from the interior; as a result of these differences in ionic concentration and the special properties of the membrane there is a resting potential, the interior of the fibre being some 60–90 mV negative to the exterior. During the impulses this potential difference is reversed so that the inside becomes about 30 mV positive. The mechanism of the impulse has been discussed fully by HODGKIN (1951); the essential point to make here is that an impulse is initiated whenever the membrane potential falls sufficiently. A reduction of about 10 per cent in the membrane potential is usually enough to initiate the regenerative processes that result in an all-or-nothing impulse. After an impulse the fibre is at first completely inexcitable, but excitability then returns over a period of a few msec. If a constant current is passed through a nerve fibre a repetitive discharge of impulses can be obtained; the frequency of this discharge is a function of the current, the intervals between impulses being determined by a number of factors (HODGKIN, 1948; KATZ, 1950b).

Impulses can be initiated by an external source of current, and they can also be initiated by any activity of the cell itself that is able to discharge the membrane capacity and reduce the membrane potential by more than the critical amount. It is known from direct evidence that certain sense organs produce potential changes which are localized in the receptor region and which are related, both in their magnitude and time course to the physical stimulus; similar potential changes have also been observed in single receptors and in these the change has been shown to be a reduction in membrane potential. These graded potentials, that precede and are believed to initiate the nerve impulses, have been termed generator potentials (BERNHARD *et al.*, 1942). Some of these generator potentials represent the activity of

single receptors and are conveniently known as receptor potentials, while others represent the activity of whole tissues.

Generator potentials have been described in the "microphonic potentials" of the mammalian cochlea (WEVER and BRAY, 1930; DAVIS *et al.*, 1952), of the lateral line organs of fishes (JIELOF *et al.*, 1952) and of the teleost sacculus (ZOTTERMAN, 1943). Of these the cochlea microphonic is the best understood; it is an alternating potential change, which has the same frequency as the applied sound pressure, and which has an amplitude related to this pressure. It is generated between two compartments, the scala media and scala tympani, and has its maximum amplitude in the region of certain special "sense cells," the hair cells, which lie in the cochlea partition; this partition is displaced by the pressure changes (V. BÉKÉSY and ROSENBLITH, 1951). Potential changes which are probably due to the synchronous activity of receptors have been found in light sensitive organs (GRANIT, 1955) and the olfactory mucous membrane of the frog (OTTOSON, 1956).

The receptor potentials of a number of receptors have now been observed; among these are single omatidia of Limulus (HARTLINE *et al.*, 1952), single receptors of the retina of the frog (SVAETICHIN, 1956) and the three mechano-receptors whose mechanism is the main subject of this article.

3. *The problem*

The problem to be discussed in this article is the mechanism by which certain mechanoreceptors produce receptor potentials in response to mechanical changes. These receptors are the muscle spindle of the frog, the Pacinian corpuscle of the cat and a stretch receptor found in crustacea. These, like all receptors, are biological transducers; there is an input which is of one energy type and an output which is of another type. In the examples to be discussed the input is some function of mechanical energy and the output, the receptor potential, is electrical in nature. There are two ways, which are of course closely interrelated, of investigating such a system; one can ignore the mechanism and simply, regarding it as a "black box," investigate its performance by working out the quantitative relationship between input and output; or one can concern oneself with the mechanism of the transducer. In this article the main emphasis will be on the latter though it will of course be necessary to deal with quantitative input–output relationships as these provide a great deal of evidence as to mechanism.

Functionally these mechanoreceptors can be divided into three parts, a mechanical system which transmits and modifies the mechanical energy, the biological transducer in which the electrical energy is developed, and an electrical system which transmits the output of the

transducer to both the site of impulse initiation and the site of recording. As a first approximation these elements may be associated structurally with the supporting tissues, the membrane of the terminal portions of the nerve fibre and its immediate surroundings, and the nerve fibre respectively. In approaching this problem the structure of the receptors and the methods used in their investigation will be considered first. This will be followed by an account of their overall behaviour and finally their mechanism will be discussed.

II. Structure

Much has been written on the general histological appearance of the structurally specialized receptors that are found in a limited number

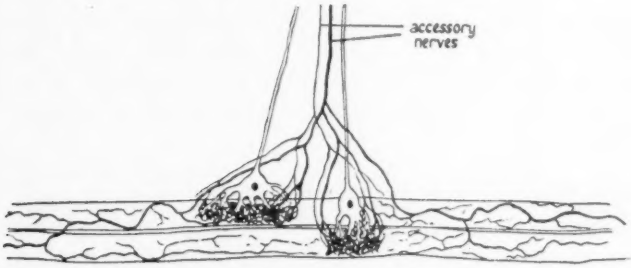

Fig. 1*a*. Diagram of the crustacean stretch receptor. From *Homarus vulgaris*. Cell 1, upper left, is "slowly adapting"; Cell 2, lower right, is "rapidly adapting." The accessory nerves do not conduct impulses set up by receptor activity. The cell bodies are of the order of 100–200μ in diameter (from Alexandrowiz, 1951 Text-fig. 8D).

of species. Whatever the functional role of this specialization it is certain that functional specificity is not dependent on it, since, taking the animal kingdom as a whole, it is probable that the great majority of receptors show no structural specificity to light microscopy while showing considerable functional specificity. It is important to emphasize this before proceeding to discuss the very marked structural specialization that has occurred in two of the three receptors under consideration. It is not surprising that detailed experiments should have first been done on structurally specialized receptors, since they constitute much more manageable entities than do the rather diffuse ramifications of the non-myelinated terminals of "structurally undifferentiated" receptors.

Illustrations of the examples to be discussed are shown in Fig. 1. The type of stretch receptor found in certain crustacea and described by Alexandrowicz (1951) is illustrated in Fig. 1*a*; these receptors, which occur in pairs, each involve a small muscle and a nerve cell.

The cell bodies of the nerve cells are, as is common in invertebrates, found in the periphery and processes of the cells ramify in the muscles.

Fig. 1*b* is a photomicrograph of a frog's muscle spindle. Each spindle is built round a special bundle of muscle fibres, the intrafusal muscle. These muscles run for distances up to 15 mm and each may have several spindles on its length (E. G. Gray, 1957). A myelinated nerve emerges from the receptor region of each spindle.

Fig. 1*c* represents a Pacinian corpuscle from a cat (Pacini, 1840; Stöhr, 1928). A central approximately straight non-myelinated nerve terminal runs up the centre of the corpuscle in a space termed the central core. This terminal is surrounded by a series of 30–70 lamellae in the form of coaxial prolate spheroids. The nerve fibre gains a myelin sheath at the point at which it passes from the central core and then, after passing through the lamellae, it emerges from one pole of the body.

In considering the structure of these receptors in more detail it will be convenient to divide the description into three parts; the general structure of the nervous elements, the fine structure of the nerve terminal and its immediate surroundings, and the structure of the supporting tissues.

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1. *The general structure of the nervous elements*

The structure of the nerve fibre is of importance because it acts as an electric cable which enables those electrical events that occur in the terminal to be transmitted both to electrodes applied at a distance and to that part of the fibre where impulses are initiated. The structure of the nerve fibre of the Pacinian corpuscle from the mesentery of the cat has been studied by Quilliam and Sato (1955). Fig. 2 summarizes their findings. There is a non-myelinated terminal having a diameter 2–3 μ and a mean length of 600 μ. The extreme end is marked by a swelling and some division, the number of branches normally being only two. At the point at which the fibre leaves the central core, that is the space inside the innermost of the lamellae, the fibre becomes myelinated; in other words the nerve fibre becomes covered with a layer of myelin, a substance having a high resistivity. The myelin sheath is broken at intervals and these breaks, called nodes of Ranvier, constitute low impedance pathways between the inside and outside of the nerve fibre; it has been shown that impulses are only actively developed at the nodes of Ranvier (Huxley and Stampfli, 1949; Tasaki, 1953). It is known that on the nerve fibre of a Pacinian corpuscle nodes occur regularly at specific points; the first (node 1) occurs approximately half-way between the end of the central core and the boundary of the corpuscle; the second (node 2) occurs at the point at which the nerve fibre leaves the corpuscle. The mean lengths of the first three sections of myelin, or internodes, are 247, 247 and

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Fig. 1*b*. Photomicrograph of muscle spindle of the frog (*Rana temporaria*) (from KATZ, 1950a Fig. 1A).

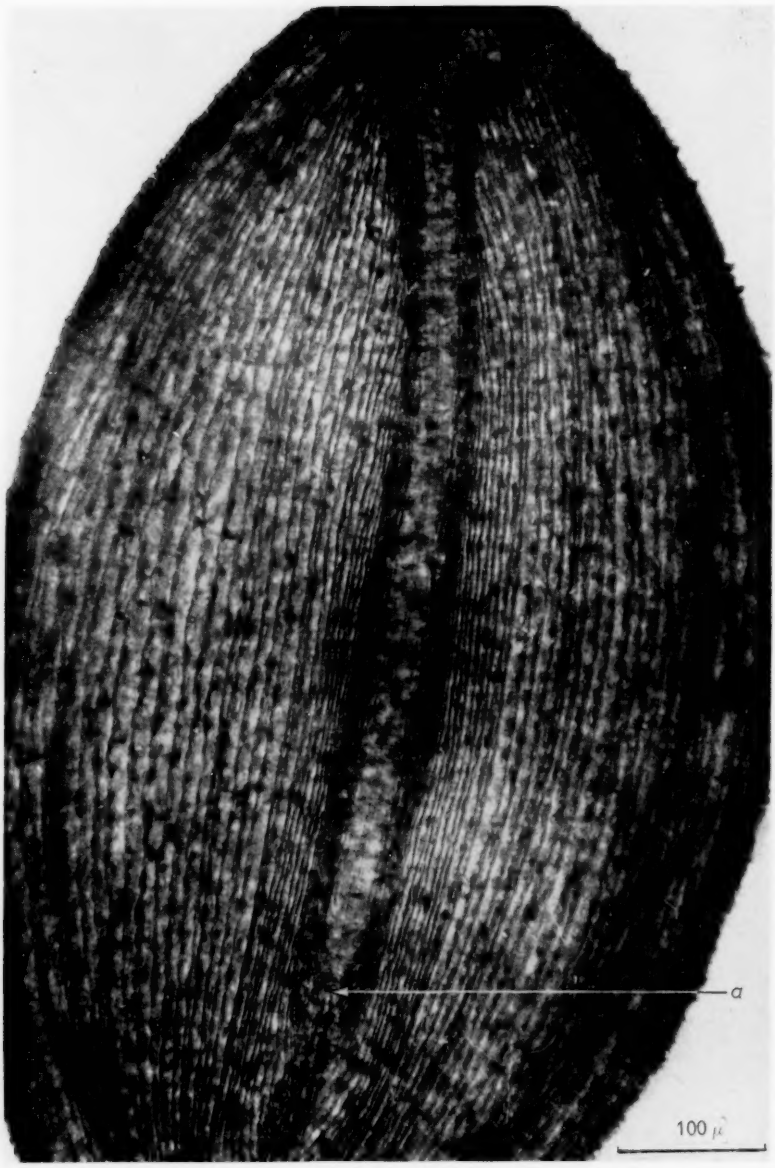

Fig. 1*c*. Flash photomicrograph of a whole Pacinian corpuscle from the domestic cat. Top, peripheral end of body. *a*, marks the end of the central core; above this the nerve fibre can be seen faintly running up the centre of the core; below this the fibre, now myelinated, appears with thicker bounding lines. The minor axis is *c*. 500μ (from HUBBARD, 1958 Plate 1).

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285 μ. It will be shown later that there is reason to suppose that the nerve impulse is initiated at the first node of Ranvier (p. 308); in many experiments on this structure records have been obtained from the region between the second and third nodes.

There are two nerve cells associated with each stretch receptor element of the type found in certain crustacea and illustrated in Fig. 1*a*. There are both structural and functional differences between the two cells; thus cell 1 (Fig. 1*a*) is broader and has more and longer

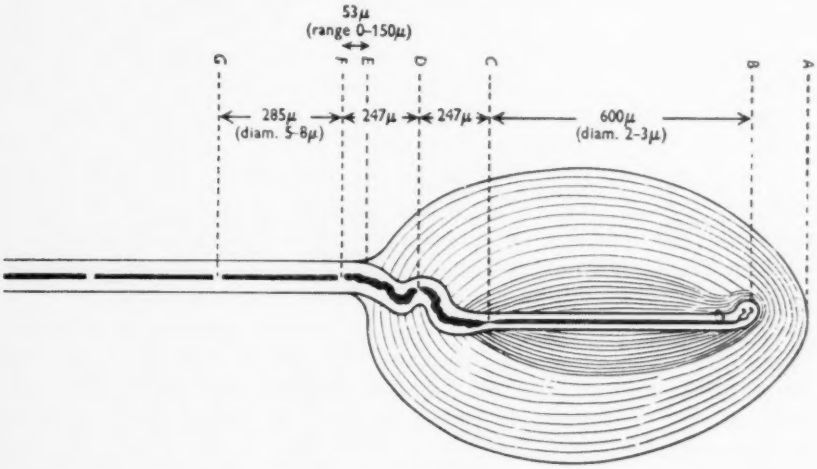

Fig. 2. Diagram of Pacinian corpuscle to show dimensions. *B* to *C* central core (central end not correctly shown, cf. Fig. 1*c*). *D*, node 1; *F*, node 2; *G*, node 3. Nerve fibre non-myelinated from *B* to *C*, myelinated central to *C* (from QUILLIAM and SATO, 1955 Fig. 4).

processes (called dendrites) than cell 2. No part of either type of cell is covered with myelin. Cells of type 1 respond with a given frequency to a given amount of stretch and have been called by WIERSMA *et al.*, 1953, "slowly adapting" or "slow" cells; on the other hand the frequency of the response of a cell of type 2 decreases during a maintained stretch and these cells they have called "rapidly adapting" or "fast" cells. Records of receptor potentials have been obtained from the cell bodies of both types of cell and there is evidence, considered below, which indicates that impulses are initiated by the "fast" cells in the cell body. The complex system of dendrites thus represents an electrical system that transmits, passively, to the cell body potential changes due to receptor activity in the terminals.

The nerve fibre from the receptor of the frog's muscle spindle is like that of the crustacean stretch receptor in that it branches extensively before it terminates; many branchings occur before the myelin sheath

is lost. These divisions are found where the nerve is winding round the intrafusal muscle, in the centre of the spindle (E. G. Gray, 1957). In this receptor, as in the crustacean stretch receptor, a complex system of fibres lies between the sites at which the receptor potentials are generated and the site from which they can be recorded.

2. *The fine structure of the terminal*

Electron-microscopical studies of the cat's Pacinian corpuscle (Pease and Quilliam, 1957) and of frog's muscle spindle (Robertson, 1957) have been made. There is one striking feature common to both types of terminal and also to the free receptor nerve terminals of the cornea (Whitear, 1957); that is they have lost their Schwann cell sheath, so that the limiting membrane of the nerve fibre itself is in direct relation with the surrounding structures.

The whole length of the fibre in the central core of the Pacinian corpuscle is without a Schwann cell sheath and is elliptical in cross section. This fibre lies in close relation to two D-shaped structures, which are divided from each other by the nerve fibre itself and by clefts which continue the long axis of the elliptical cross-section of the fibre (Fig. 3*a*). These D-shaped structures appear to be composed of interlacing cytoplasmic processes of cells whose nuclei lie around the periphery of the central core. The limiting membranes of the innermost part of these structures lie in close relation to the nerve fibre membrane except at the base of the clefts.

The nerve fibres in the frog's muscle spindle, before they lose their Schwann cell sheaths, lie in close relation to the intrafusal muscle fibres (Fig. 3*b*); as the ending is approached, however, the nerve fibre loses its Schwann cell and at about the same point leaves the muscle fibre to end in the undifferentiated ground substance.

The terminations of these nerve fibres, and also those of the corneal fibres, are particularly rich in mitochondria; those found in the terminal of the nerve fibre in the Pacinian corpuscle are arranged in a regular pallisade around the periphery; in the other instances the arrangement is irregular. The fibre of the Pacinian corpuscle also contains vesicles.

An absence of the Schwann cell sheath and large numbers of mitochondria and vesicles have also been found in the nerve terminals associated with the hair cells of the mammalian cochlea (Smith and Dempsey, 1957).

3. *Supporting tissues*

The mechanical properties of the supporting tissues can be of great importance to the behaviour of the organ. This is particularly clear in the case of the Pacinian corpuscle which has been investigated more

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Fig. 3*a*. Diagram of the central region of a Pacinian corpuscle as seen in transverse section. In the centre, the nerve fibre (major axis *c.* 3μ), without myelin, but showing a pallisade of mitochondria. Outside this is the zone with cytoplasmic processes divided into two groups by the cleft that lies along the plane of the major semi-axis of the section of the fibre. Outside this the cell bodies connected with the cytoplasmic processes and on the outside of the diagram the innermost lamellae (from PEASE and QUILLIAM, 1957 Text-fig. 1).

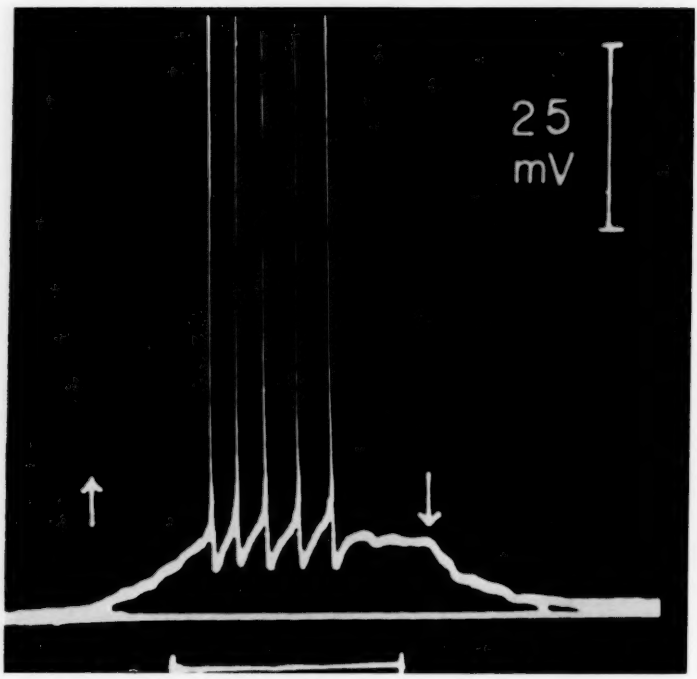

Fig. 5. Receptor potential with superimposed nerve impulses from a stretch receptor of the crayfish ("slow" cell). Arrows mark duration of stretch; time, 1 sec (from EYZAGUIRRE and KUFFLER, 1955a Fig. 4*a* right).

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fully, in this respect, than other receptors. A large Pacinian corpuscle is about 1 mm in length and 0·6 mm in width and has a mass about 0·15 mg wet and 0·02 mg dry (GRAY and HUBBARD, unpublished); it is built up of a considerable number of coaxial lamellae. Each of these lamellae consists of a thin protoplasmic sheet derived from a few cells (PEASE and QUILLIAM, 1957); the junctions between the cells are

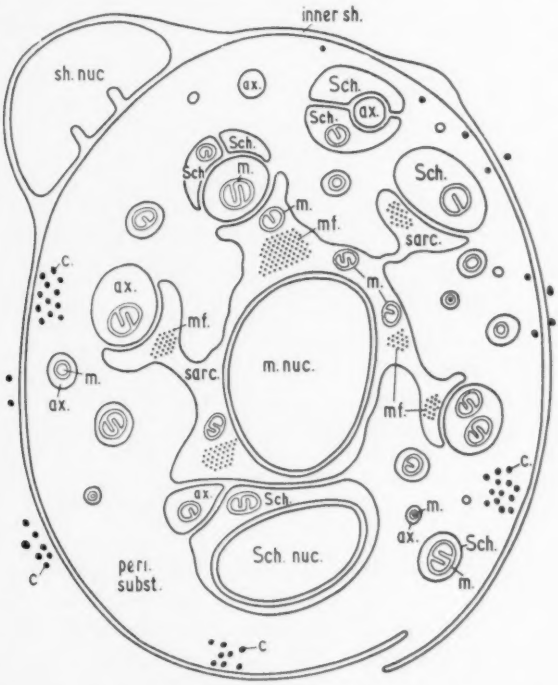

Fig. 3*b*. Diagram of a cross-section of a portion of a frog's muscle spindle, at resting length, in the region of the receptor innervation. inner sh., sh.nuc, intrafusal muscle fibre inner sheath and its nucleus; m.nuc., muscle nuclei; m.f, myofilaments; sarc., sarcoplasm; m., mitochondria; peri.subst., perimuscular substance; ax., nerve fibres; Sch., Schwann cells (by courtesy of J. D. ROBERTSON).

close and overlapped. The thickness of this sheet is small, only about 0·2 μ, but it is supported by collagen fibres that run on both sides in the plane at right angles to the long axis of the corpuscle. These lamellae, which have a low permeability to certain ions (GRAY and SATO, 1955), completely separate spaces containing liquid; this liquid can be seen to run out when the outer lamellae are divided (PACINI, 1840). The spacing of the lamellae is related to the distance from the axis, and appears to be logarithmic (HUBBARD, 1958); i.e. if D_n is the diameter of the nth lamella in cross-section and D_0 and γ are constants,

$\log D_n = \log D_0 + \log \gamma$. The spacing nearly always shows one and sometimes two abrupt changes, which can be represented by changes in the value of γ; these discontinuities in the spacing are associated with discontinuities in the mechanical behaviour (p. 305).

The supporting tissues of the crustacean stretch receptor and the frog's muscle spindle are more complicated and have not yet been investigated from a mechanical point of view. The main complicating factor is that in both instances much of the support is provided by muscle which is an active tissue.

III. Methods

1. *Recording*

In all the examples discussed in this article the electrical changes are developed at a site which is difficult of access, and records of the changes in potential have been obtained by placing electrodes at a distance.

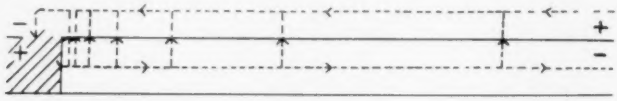

Fig. 4. Diagram to illustrate distribution of current in a section of a non-myelinated nerve fibre immediately adjacent to a region in which the membrane potential has been altered. Shaded area, region of potential alteration; dotted lines indicate direction and density of current.

Since a nerve fibre consists of a conducting core bounded by a cell membrane that has a high transverse resistance and the whole is immersed in a conducting medium, it behaves like an electric cable. Fig. 4 shows in a generalized way the flow of current that occurs in a nerve fibre as a result of a change in the potential across part of the nerve membrane; the diagram represents events in a non-myelinated fibre in which case the decrement of current density along the fibre is continuous; in the case of the myelinated fibre a considerable proportion of the current leaves the fibre at nodes of Ranvier. An analysis of the cable-like properties of non-myelinated nerve fibres has been made by Hodgkin and Rushton (1946). They found that the space constant, which represents the distance from a steady source of potential at which the membrane potential is 1/e of the membrane potential at the source, had values of the order of a few mm; membrane time constants were of the order of a few msec. A review of the electrical constants of nerve membranes has been given by Katz (1952).

In the experiments on the crustacean receptor (in the majority the crayfish, *Procambarus alleni* (Faxon) was used) a micro-electrode was inserted into the cell body (Fig. 1*a*) and the potential across the

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membrane recorded with an instrument having a very high input impedance; a receptor potential appeared positive with respect to the internal electrode. In most of the other experiments, to which reference is made, the current flowing external to a particular section of the fibre has been recorded. In general this has been done by dissecting the fibre as cleanly, and as close to the ending, as possible and then providing a high external resistance by mounting the fibre in an insulating medium, e.g. paraffin oil. Potential records have been made by means of electrodes placed on either side of the resistance. Receptor potentials cause negativity of the electrode nearest the receptor. The preparations have all been isolated and investigated at room temperature.

2. *Stimulating*

In experiments in which the time course of the mechanical displacement has been important, either piezo-electric crystals or electromagnetic transducers have been used to cause the displacement. The former have been used when a rapid onset has been required, while the electromagnetic methods have been preferred when large movements have been needed. Records of the output of these transducers have been obtained photoelectrically or by means of a recording transducer.

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IV. Properties of Receptor Potentials

1. *General description*

Receptor potentials are reductions of membrane potential which are localized in the receptor region, and whose amplitude and other properties are related quantitatively to the stimulus. Their time course has been found to vary considerably from one preparation to another. The "slowly adapting" type of stretch receptor found in the crayfish (Eyzaguirre and Kuffler, 1955a) exhibits a receptor potential that is maintained at a steady level as long as a constant stretch is applied to the associated muscle (Fig. 5). The maximum duration of a receptor potential in a published figure is about 10 sec, but studies of impulse activity in this and other mechanoreceptors indicate that receptor potentials can be maintained for long periods. The record illustrated in Fig. 5 shows not only the receptor potential but also all-or-nothing nerve impulses which are being excited by it. The presence of all-or-nothing impulses tends to obscure the receptor potentials and therefore impulse activity has often been prevented by one means or another; most of the material discussed here has been obtained from such preparations.

The long maintained receptor potential of the "slowly adapting" stretch receptor is associated with its ability to maintain a constant

frequency of impulses. In the "rapidly adapting" type of stretch receptor the impulse frequency declines during a maintained stretch. EYZAGUIRRE and KUFFLER (1955a) have shown that this is due to the inability of this receptor to maintain a steady receptor potential; its amplitude declines, over a period of seconds, during a steady stretch. The effects on the receptor potentials of rapid changes in length of these receptors have not yet been described.

The receptor potential of the frog's muscle spindle has been shown by KATZ (1950b) to consist of three parts: the dynamic component, the static component and the positive deflexion on relaxation (Fig. 6). The dynamic component is a peak, which occurs during the application of a stretch, and of which the amplitude depends on the velocity as well as on the extent of the displacement. The static component, which follows the dynamic component, is maintained at a constant level as long as the stretch is kept constant and is independent of the stimulus velocity. Finally when the receptor is relaxed a potential change of opposite polarity occurs. These three phases of the receptor potential correspond to three phases in the pattern of the impulse discharge from the receptor (MATTHEWS, 1931a, b); first there is a high frequency burst associated with the change of length, then a lower frequency maintained throughout the stretch, and finally, when the stretch is released, the frequency is reduced below its original resting level or even abolished altogether.

The receptor potentials recorded from Pacinian corpuscles (ALVAREZ–BUYLLA and RAMIREZ DE ARELLANO, 1953; GRAY and SATO, 1953, cf. GRAY and MALCOLM, 1950) are in some respects different from those just described. The potential reaches a maximum in 1–2 msecs (at room temperature) and immediately decays with a time course that is consistent with an exponential (Fig. 7); the mean value of the time constant of decay given by GRAY and SATO is 1·7 msec (four experiments, range 1·2–2·4 msec). These receptor potentials always represent reductions of membrane potential and the same response is observed both at the beginning and at the end of a long compression or resulting from a short mechanical pulse. No change of potential can be seen during a maintained compression, except for the transient changes (Fig. 7) that occur at the beginning and end. The sensitivity to the onset of a compression is usually greater than that to the following decompression. However, the difference is not usually great and sometimes the sensitivities are reversed.

2. *Effects of the extent of displacement*

The amplitude of a receptor potential depends on the extent of the exciting displacement, whether it be a lengthening as in the case of the muscle spindle or a compression as with the Pacinian corpuscle. The

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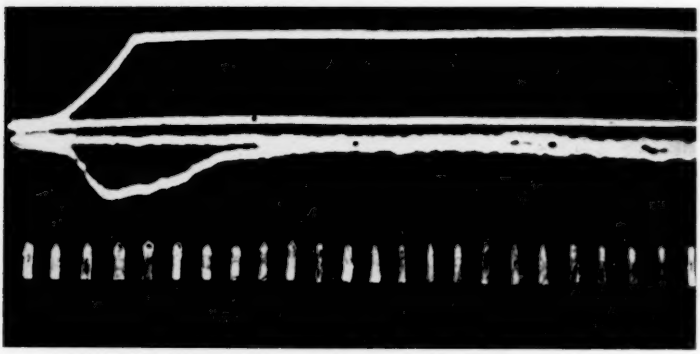
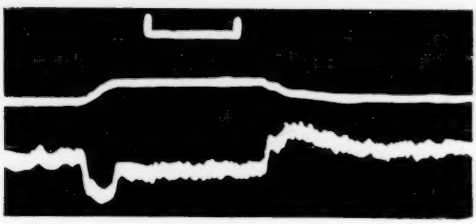


(*a*)



(*b*)

Fig. 6. Receptor potentials from the frog's muscle spindle. *a*, to show onset of stretch; top pair of traces, stretch; bottom pair, receptor potential; time, 500/sec. *b*, to show stretch and relaxation; top, stretch; bottom, receptor potential; time 0·1 sec (from KATZ, 1950b Fig. 6c, top and Fig. 13B3).

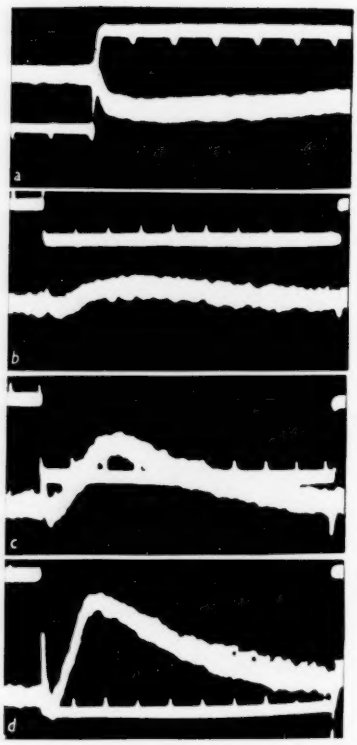


Fig. 7. Receptor potentials from a Pacinian corpuscle. *b–d*, top beams (at left) indicate amplitude and time course of displacement and time msec; bottom beams, receptor potentials. *a*, bottom beam (at left) voltage applied to crystal stimulator (same as top beam in other records though with smaller amplification) and time msec; top beam, displacement recorded photoelectrically. Records of superimposed traces (from GRAY and SATO, 1953 Fig. 7).

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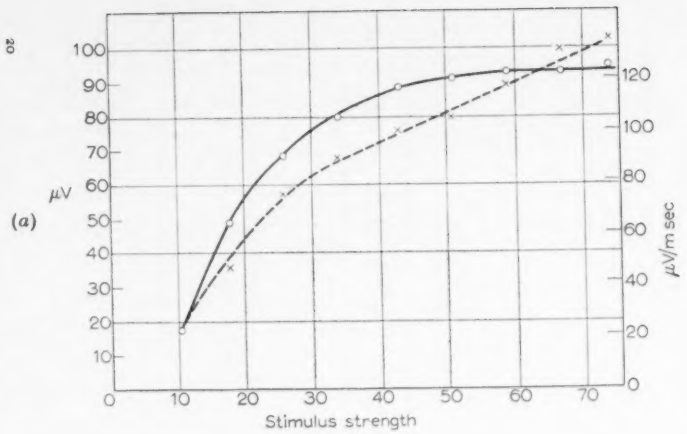

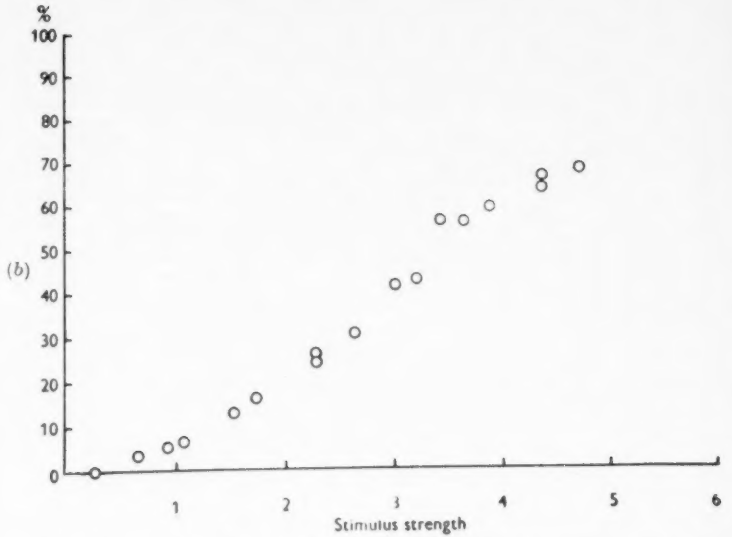

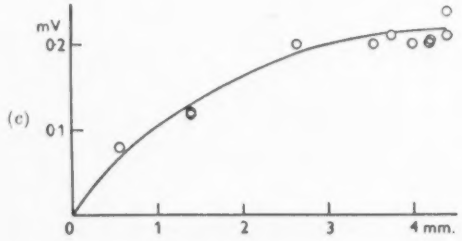

Fig. 8. Quantitative effects of changes in displacement amplitude. (*a*) Pacinian corpuscle: 0————0; abscissa, relative displacement (10 units = 5·8 V applied to crystal); ordinate, receptor potential amplitude μV; means of 8 observations, SE's of means *c.* 1 μV. X - - - - X; abscissa, volts applied to crystal; ordinate, rate of rise of receptor potential μV/msec. (*b*) another Pacinian corpuscle: abscissa, relative displacement as volts applied to crystal; ordinate, receptor potential amplitude as percentage of maximum; small values of displacement only illustrated. (*c*) frog's muscle spindle: abscissa, stretch mm; ordinate, receptor potential amplitude mV ((*a*) from Diamond *et al.*, 1958a Fig. 4*d*; (*b*) from Gray and Sato, 1953, Fig. 8, circles only; (*c*) from Katz, 1950b Fig. 9).

three full curves in Fig. 8 illustrate this point. Fig. 8(*a*) records the mean of a series of observations on a Pacinian corpuscle and shows that with increasing compressions the receptor potential amplitude increases until a maximum value is approached. Fig. 8(*b*) is a series from another corpuscle to show in more detail the lower part of the curve; it can be seen that a minimum amount of compression must occur before any change of potential can be detected and that in its lower part the curve is concave upwards. The abscissae of these graphs may be calibrated in volts applied to the crystal transducer; relative values in any one experiment are accurately represented in this way, but absolute values and comparisons between experiments are not reliable. The order of magnitude of the displacements can, however, be indicated; one volt represents approximately 0·2–0·5 μ and a receptor potential 90 per cent of maximum is obtained with a stimulus of about 2–5 μ. Results obtained from a frog's muscle spindle are plotted in Fig. 8(*c*) which relates amplitude of the static phase of the receptor potential to the amount of stretch; it can be seen that this curve is very similar to that shown in Fig. 8(*a*).

The amplitude of the receptor potential is not the only parameter which changes with displacement amplitude. The rate of rise of the potential also increases with increasing displacement. The relation between the maximum rate of rise of the potential and the compression is shown by the dotted line in Fig. 8(*a*), which is from the same preparation as the full line in the same section of the figure. It can be seen that the rate of rise continues to increase at values of compression at which the receptor potential amplitude is virtually maximal. It is a corollary that this change in the rate of rise of the potential must be accompanied by a change in its time of rise; such a change has been described by Gray and Sato (1953).

3. *Effects of the velocity of displacement*

The amplitudes of receptor potentials are also dependent on the velocity of the displacement. This was first shown by Katz (1950b) for the dynamic phase of the receptor potential of the frog's muscle spindle. The relation he obtained is shown in Fig. 9*a*. A similar relation (Fig. 9*b*) has been found for the receptor potential of the Pacinian corpuscle (Gray and Sato, 1953). In both preparations velocities greater than a certain value gave a response of maximum amplitude while at lower velocities the amplitude declined as the velocity of stimulus decreased. In the muscle spindle the receptor potential amplitude is related to velocity at values below 200 μ/msec. The corresponding value for the Pacinian corpuscle is of the order of 1–2 μ/msec. These figures are quoted to show that the relation to velocity is likely to be functionally important; a comparison of the two figures should not, however, be

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made because a maximum receptor potential of the Pacinian corpuscle is obtained with a displacement of 2–5 μ (i.e. *c.* 0·4–1 per cent) while a maximum static component of the spindle potential is only obtained with a stretch of the muscle of 3000–4000 μ (i.e. *c.* 15–20 per cent).

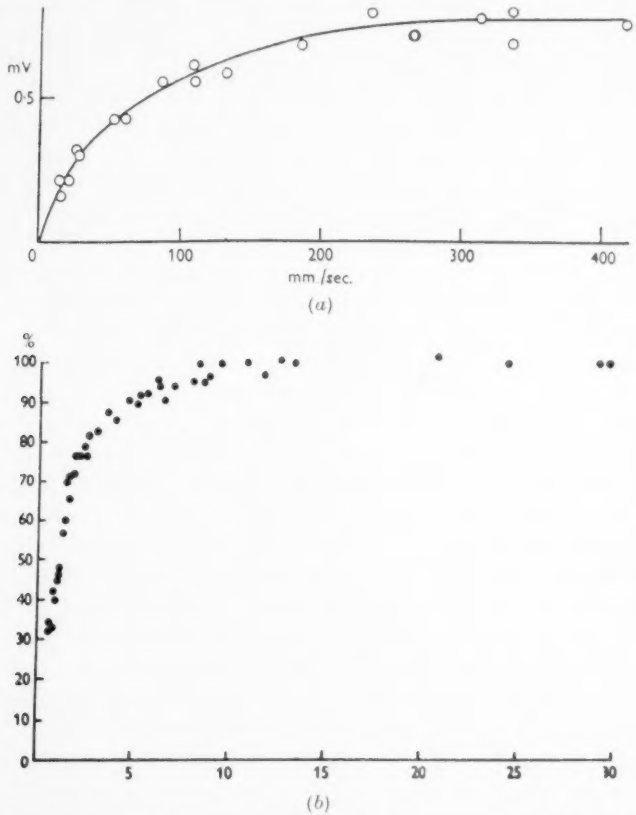

Fig. 9. Quantitative effects of velocity of displacement. (*a*) frog's muscle spindle: abscissa, velocity of stretch mm/sec; ordinate, amplitude of dynamic phase mV. (*b*) Pacinian corpuscle: abscissa, relative velocity of displacement as volts applied to the crystal per msec; ordinate, amplitude of receptor potential as percentage of that with displacement of infinite velocity (*a*) from Katz, 1950b Fig. 8a; (*b*) from Gray and Sato, 1953 Fig. 11).

The great differences in these figures are probably not related to any difference in the sensitivities of the biological transducers themselves, but to differences in the mechanics of the two systems.

4. *Absolute size of receptor potentials*

Estimates of the absolute size of receptor potentials have been made from indirect evidence for both Pacinian corpuscles and the crayfish

stretch receptor. The receptor activity of Pacinian corpuscles was recorded as the current flowing externally between the second and third nodes of Ranvier (DIAMOND *et al.*, 1956), (for numbering of nodes, see Fig. 2 and Fig. 18). The current caused by a maximum receptor potential was compared with that caused by impulse activity both at the second and at the third node of Ranvier. The change in current due to the receptor potential was 59 per cent ($n = 6$; S.D. = 14 per cent) of that due to an impulse at node 2, and 38 per cent ($n = 5$; S.D. = 17 per cent) of that due to an impulse at node 3. The difference in the two figures is probably due to differences in the physiological state of the nodes rather than to a physical asymmetry. The receptor potential is attenuated between the non-myelinated terminal and the second node (cf. Fig. 18). We have no direct information regarding the size of this attenuation; TASAKI (1953) gives a factor of 0·5 per internode for large myelinated fibres of the toad, but the attenuation between nodes may be less in the case under discussion. A factor of 0·5 for two internodes might be a reasonable estimate. Doubling the figures quoted above would indicate that a maximum receptor potential is of the same size as an impulse; the impulse is normally 20–30 per cent greater than the resting potential. There are reasons for thinking that even in the intact animal the resting potential of the nerve fibre near this ending is low and the impulses small; under these conditions the impulse would be expected to be nearer the size of the resting potential. In spite of the uncertainties of this estimate, one can say that a maximum receptor potential causes a potential change to occur across the membrane of the nerve fibre terminal which is not less than half the membrane potential or more than twice the impulse and is therefore of the same order of magnitude as the resting potential and impulse.

The estimates made by EYZAGUIRRE and KUFFLER (1955a) of the amplitude of the crayfish stretch receptor potentials refer to threshold values rather than maximum values of receptor potential. They recorded threshold values of about 8 mV for "slowly adapting" cells and 25 mV for "rapidly adapting" cells. They estimate that these recorded values are 20–80 per cent of those at the terminals.

5. *Summation*

Receptor potentials will add together if two or more occur at the same time. When considering maintained receptor potentials, this means that successive steps of displacement are each accompanied by a change of potential to a new level (Fig. 10). Summation can also occur when there is no summation of the stimulus; receptor potentials of Pacinian corpuscles have been shown to summate, even when excited by short pulses that are independent of each other (ALVAREZ–BUYLLA and RAMIREZ DE ARELLANO, 1953; GRAY and SATO, 1953) (Fig. 11*a*). It

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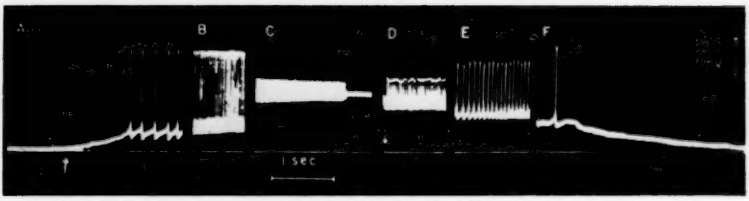

Fig.10. Receptor potential and impulses from a "slow" cell of a crayfish stretch receptor. Increasing stretch by steps (from EYZAGUIRRE and KUFFLER, 1955a, Fig. 9).

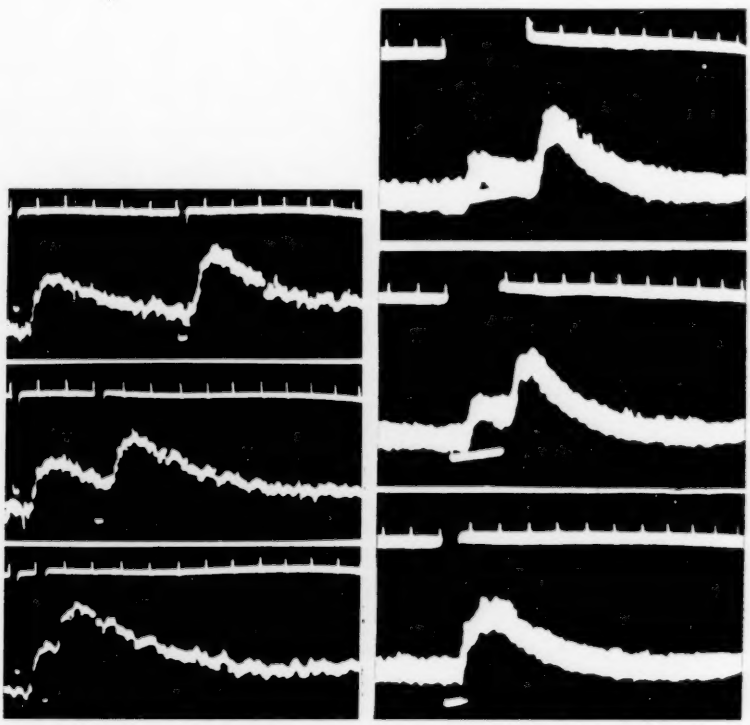

Fig. 11. Summation of receptor potentials of Pacinian corpuscles. Top beams indicate amplitude and time course of exciting displacements and time msec. Bottom beams record receptor potentials. *a*, receptor potentials excited by short pulses at three different intervals. *b*, receptor potentials excited by the "on" and "off" of a single pulse at three different durations; records of superimposed traces (from GRAY and SATO, 1953; *a*, Fig. 13; *b*, Fig. 14).

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has already been pointed out that in this ending brief receptor potentials, which always have the same polarity, occur both at the onset and release of a compression. Two receptor potentials set up by the compression and decompression of a short pulse can also summate (Fig. 11*b*). It has been clearly shown by ALVAREZ–BUYLLA and and RAMIREZ DE ARELLANO that this property can be of functional significance since repeated short pulses, i.e. vibrations, can excite when a single pulse cannot. The magnitude of the effect is, however, not as great as might be expected from our knowledge of the similar summation

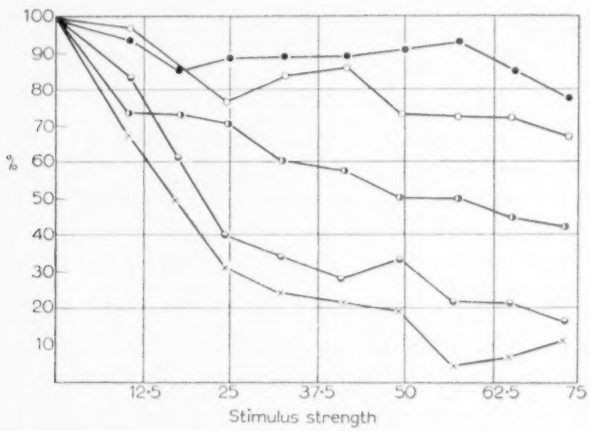

Fig. 12. Depression curves from a Pacinian corpuscle. Abscissa, relative amplitude of conditioning (first) stimulus, (10 units = 5·8 V applied to the crystal); ordinate, test receptor potential amplitude as percentage of amplitude without a conditioning stimulus. Each curve for a particular stimulus interval: ×, 1 msec; ○, 2 msec; ◑, 4 msec; ○, 6 msec; ●, 7 msec. Same preparation as Fig. 8*a* (from DIAMOND *et al.*, 1958a, Fig. 3).

found with end-plate potentials (ECCLES *et al.*, 1941) and synaptic potentials (BROCK *et al.*, 1952); the difference is due to the depression that follows each receptor potential.

6. *Depression*

The amplitude of a receptor potential set up in a Pacinian corpuscle within about 10 msec of another receptor potential is decreased (GRAY and SATO, 1953; LOEWENSTEIN and ALTAMIRANO–ORREGO, 1958; DIAMOND *et al.*, 1958a). This depression increases as the amplitude of the first receptor potential increases and as the interval between the two responses becomes shorter (Fig. 12). Depression can also be caused by an impulse though the effect is small compared with that produced by a maximum receptor potential (DIAMOND *et al.*, 1958a).

The explanation of depression is by no means clear. There is good evidence that it is not a mechanical phenomenon (DIAMOND *et al.*, 1958a) and some evidence suggests that it must, in part, be related to the transient change of potential across the terminal membrane. However, it is possible to increase the degree of depression by increasing the strength of the first stimulus at values at which little or no change in the amplitude of the first receptor potential occurs but at which the rate of rise of this potential change is still increasing (cf. Figs. 8a and 12); this result suggests that other processes may be involved. The problem is considered again later in this article (p. 315), after the mechanism of the receptor has been discussed.

V. MECHANISM

An account has been given of the structure and overall behaviour of certain mechanoreceptors. It is now possible to consider the main problem; the mechanism of the receptors. This part of the article is divided into three main parts: on the mechanics of the body, on the site and nature of the biological transducer, and on the electrical characteristics of the output.

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1. *Mechanical transformations*

In experiments on receptors such as those already described the applied mechanical deformations were measured at the output of the stimulator. The mechanical properties of the receptor and its surrounding tissues may, however, modify both the amplitude and time course of the mechanical events; thus the mechanical changes to which the biological transducer is subjected may be very different from those applied externally.

EYZAGUIRRE and KUFFLER (1955a) suggested that the difference between the time courses of the receptor potentials of the "slowly adapting" and of the "fast adapting" stretch receptors of the crayfish might be accounted for by differences in the mechanical connexions between the nerve cell processes and the associated muscles. Differences in the detailed relationships of these structures have been observed histologically and they suggest that a degree of slip might occur with the "fast cells."

LOEWENSTEIN (1956) showed that the duration of a discharge of impulses resulting from a constant force applied to frog skin could be modified by mechanical means. Stretching the skin in a uniform manner caused an increase in the duration of such a discharge. Stretching mesentery containing Pacinian corpuscles (ALVAREZ-BUYLLA and RAMIREZ DE ARELLANO, 1953; LOEWENSTEIN, 1956) caused these receptors to discharge a short train of impulses in response to a long

and a maintained or static component, which is not visible in this region. This dynamic component was the only displacement that could be detected in the region of the central core; its time course was comparable with the time course of the recorded receptor potential; and it changed in a way similar to the receptor potential in response to changes in amplitude and velocity of compression. It, therefore, seems

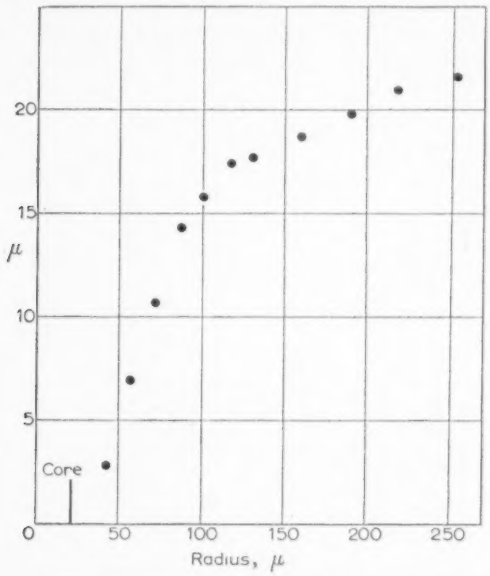

Fig. 14. Relationship of the dynamic component of lamellar displacement in a Pacinian corpuscle to radius of the lamella in cross-section. Abscissa, radius μ; ordinate, displacement μ (from HUBBARD, 1958 Fig. 14 right).

reasonable to regard this dynamic component as a precursor of the electrical process.

HUBBARD has analysed this dynamic component further. He measured the static displacement of a particular lamella for various degrees of compression at a time which, for practical purposes, could be regarded as infinity; a single value of displacement for each value of compression was thus obtained for each lamella. The time course of the compression applied in experiments such as that illustrated in Fig. 13(*a*) was known and each value of compression could therefore be converted to a corresponding value of static displacement; he thus obtained curves, such as those shown in Fig. 13(*b*), which relate equivalent static displacement to time. These curves represent what would be the behaviour of the system if it contained no elements reacting to time functions of the applied compression. By subtracting the curves obtained in this way (Fig. 13(*b*)) from the experimental curves (Fig. 13(*a*))

compression; the usual response is a single impulse discharged at the beginning and another at the end of such a compression.

HUBBARD (1958) has investigated the mechanics of the Pacinian corpuscle. His method was to take photomicrographs (Fig. 1*c*) with a flash lasting a few μsec at various times during compression of a corpuscle; the optical axis of the system was parallel to the direction of compression. By recording the time course of the compression and triggering the flash at a series of known times it was possible for him to plot the time course of displacement of any individual lamella;

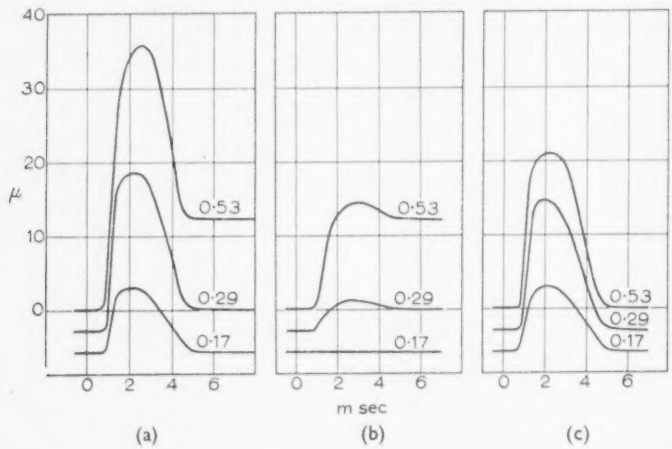

Fig. 13. Lamellar displacements in a Pacinian corpuscle. Abscissae, time msec; ordinates, displacement μ (note displacement of zero lines). Top curves for a lamella having a cross-sectional radius 0·53 of that of the outside of the corpuscle; middle curves for a radius of 0·29; bottom curves for a radius of 0·17. (*a*) observed displacements during a compression that rose approximately linearly to 62 μ in 2·3 msec. (*b*) equivalent static displacements (see text). (*c*) dynamic component (from HUBBARD, 1958 Fig. 13).

compressions having a range of amplitudes and velocities were used. He was unable to detect any changes in length or bending of the axon, but observed quite large radial displacements, which were uniform over the length of the central core. The time courses of the displacements of three different lamellae are shown in Fig. 13(*a*). The outermost of these (its radius in cross-section was 0·53 of the radius of the corpuscle) showed a relatively large initial displacement, which after a few msec declined to a steady level; this steady level was shown to be maintained as long as the corpuscle was compressed. The innermost of the three (radius 0·17) showed the rapid early displacement, but after 4–5 msec no displacement could be detected. These records show that there is an initial or dynamic component lasting a few msec, which can be detected near the outer boundary of the central core,

(the spindle efferent), causes an increase in the activity of the receptor. These spindle efferents are connected centrally to many parts of the nervous system, which is thus able to modify the activity of the receptor (KUFFLER *et al.*, 1951). A full review of this system is given by GRANIT (1955). There are other examples of the central control of receptors and not all of these are mechanoreceptors; a discussion of such instances would be out of place here.

2. *The biological transducer*

2.1. *Site.* The fact that a receptor potential can be recorded from the nerve fibre at a distance from the receptor indicates that a change of potential occurs across the membrane of the nerve fibre itself. This change of potential could be due to an active process occurring in the membrane of the nerve fibre, but it could also result from an outward* flowing current set up by activity in some other cell. There are reasons for thinking that the potential change is actively developed across the membrane of the nerve fibre itself, though these cannot be regarded as in any way conclusive. No structure that would seem suitable as an external source of current has been identified in any of these receptors. In the two muscle receptors the fine nerve terminals lie in close relation to muscle fibres; muscle fibres are known to exhibit electrical activity in connexion with the contraction of the muscle and it is difficult to see how one membrane could subserve two such different functions. In the Pacinian corpuscle the only cells that are closely related to the nerve fibre are those whose processes form the D-shaped structures in the central core. If current from these cells were to cause a change in the membrane potential of the nerve fibre, the direction of flow would have to be longitudinal to the terminal; transverse current which entered and left the fibre at one particular level could not, *per se*, cause a change in membrane potential because of the negligible transverse internal resistance. The division of the cells in question into processes and the existence of the clefts would appear to provide low resistance pathways in parallel with the fibre, and such a structure would suggest that the electrical coupling between the processes and the nerve fibre is poor. Furthermore the only displacement that has been detected near the central core is radial and uniform over the length of the core (p. 303); this arrangement does not suggest that a longitudinal current is developed. But, if it were, it would have to be sufficient to cause a change of membrane potential of the order of magnitude of the resting potential of the nerve cell. Such a potential change could only occur if the electrical coupling were extremely good, which seems improbable or if the potential change generated by the external source were

* An explanation with inward current can be devised, but is unlikely in view of LOEWENSTEIN and RATHKAMP *J. gen. Physiol.*, **41**, 1245.

he obtained curves of the dynamic component (Fig. 13(*c*)); it is clear that the lowest of the three curves is the same as the experimental curve because at this position there was no static component. It can be seen that the dynamic component decreases in amplitude as the radius of the lamella gets smaller. Larger series of observations show that the rate of decrease is comparatively small in the outer parts of the corpuscle, but increases nearer the centre. Fig. 14 illustrates the relation between the dynamic displacement and the radius of the lamella measured; the discontinuity in this curve corresponds to an abrupt change in the spacing of the lamellae of the type described in the section on structure, i.e. a change in the value of γ (p. 294). The time course of the dynamic component has not yet been investigated in detail, but it is clear from Fig. 13(*c*) that no major change is associated with its progress through the structure of the corpuscle.

The amplitude of the dynamic component of the displacement is reduced when either the amplitude of compression or the velocity of compression is reduced. Quantitative information about the relationships of the various parameters of the applied compression to the various parameters of the displacement observed at the boundary of the central core is not yet available. The anatomy of the structure (p. 293) indicates that we are dealing with a network made up of a series of elements each having essentially the same structure. Each consists of a mass of fluid bounded by membranes which may be expected to have some elastic properties. The dimensions are known and the density of the whole body is near unity; there is at present no information about the mechanical properties of the lamellae. The general type of structure is such as might be expected to behave in the manner found; however, until the necessary mechanical constants have been obtained, it will not be possible to attempt a quantitative solution of the problem.

The mechanical properties that have been considered are passive ones. There are, however, biologically active supporting tissues in association with certain receptors, and though the properties of these tissues do not fall within the line of argument of this article, some mention of them should be made. The example that is best understood is the muscle spindle of the cat (in certain important respects this end-organ differs from the muscle spindle of the frog, the receptor potentials of which have been described here). This end-organ is complex and contains two receptors and its own muscle fibres. The primary receptor is connected in series with these intrafusal muscle fibres, but the whole spindle lies in parallel with the main bulk of the muscle. Shortening of the main muscle relaxes the spindle and reduces the frequency of impulses from the primary receptor; lengthening increases the frequency (MATTHEWS, 1933). Contraction of the intrafusal muscle fibre, which is brought about by activity in its own motor nerve fibre

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considerably greater than any potential change at present known to occur in any single cell.

Loewenstein and Rathkamp (1957) have recently published a brief note of some experiments which may prove directly that the receptor potentials of the Pacinian corpuscle are not generated outside the nerve fibre. They report that the receptor potentials are not recordable after nerve degeneration. Unfortunately they do not describe their method of recording; it is clear that the usual methods of recording from this preparation could not be used for this experiment.

A comparison with other types of receptor is of little help in this connexion. There are many receptors which respond to mechanical events and which appear to be simple nerve endings having no special supporting structures whatever. On the other hand there are separate non-nervous cells, the hair cells, in the lateral line, labyrinth and cochlea; these cells are almost certainly associated with the microphonic potentials produced by these organs (Kuiper, 1956; Davis, 1957).

If it is assumed that the receptor potentials of the mechanoreceptors under consideration are generated in the nerve terminals, it is of some interest to consider what extent of the fibre is involved in this process. Eyzaguirre and Kuffler (1955a) investigated the effects of distorting the cell body of the crustacean receptor preparation and its larger processes; they were unable to excite those parts of the cell that lay outside the muscle. There is some reason to think that receptor potentials and impulses are generated in different parts of the nerve fibre (see p. 314); if this is so, information about the occurrence of impulses is relevant. Katz (1950a) demonstrated small all-or-nothing potentials in the frog's muscle spindle. These potential changes could not be observed when the receptor was discharging full-sized impulses at a moderate frequency and they could not be seen for a definite interval after an impulse had been conducted antidromically into the receptor. The small all-or-nothing potentials were probably impulses confined to branches of the nerve fibre. In the Pacinian corpuscle impulses appear to be initiated at the first node of Ranvier (see p. 308). There is, therefore, a suggestion that the region associated with receptor potentials in the spindle is confined to the smaller branches of the nerve and in the Pacinian corpuscle to the non-myelinated terminal.

2.2. *Specialization of the terminal.* There are four ways in which the terminations of receptor nerve fibres have been found to differ from other parts of the fibre. First they have been seen with the electron-microscope to differ structurally; second, they do not conduct all-or-nothing impulses; third, they are sensitive to certain chemical agents; fourth, they are highly sensitive to the physical stimulus. These special features have all been found in a number of receptors, but for no receptor have all four been described. There is no evidence that all

these forms of specialization are related, or even that they occur in identical areas of membrane. The best correlation available at the moment is that in the Pacinian corpuscle; the non-myelinated terminal shows special structural features throughout its length and it is this part of the nerve that does not conduct all-or-nothing impulses.

2.2*a*. *Structural specialization.* Structural specialization has already been considered in an earlier section; the most striking features are, perhaps, the loss of the Schwann cell by the terminal and the large number of mitochondria. Both these features have also been found in the nerve fibres associated with hair cells.

2.2*b*. *Absence of impulses.* There is evidence from both the stretch receptor of the crayfish and the Pacinian corpuscle of the cat that neither are mechanically excited all-or-nothing impulses initiated in the terminations of the nerve fibre nor are they invaded by antidromically conducted impulses.

The "rapidly adapting" type of receptor in the crayfish initiates nerve impulses across the membrane of, or near, the cell body (Eyzaguirre and Kuffler, 1955a). This was shown by measuring the amplitude of that receptor potential, recorded from the cell body, which was just threshold for the initiation of an impulse; it was found to be the same size as a threshold change of membrane potential produced by other means. If the receptor potential initiated impulses at a distance from the cell body, i.e. in the terminals in the muscle, the apparent size of the receptor potential at threshold would have been less than the critical potential of the cell membrane because of the attenuation introduced by the intervening nerve fibre (see p. 294). Such a result was in fact obtained by Eyzaguirre and Kuffler (1955a) for the "slowly adapting" receptor; in this receptor cell the dendrites are longer than in the "rapidly adapting" type and the impulse appears to be initiated at a distance from the cell body. Case *et al.* (1957) have now produced evidence that the impulse may be initiated in the beginning of the axon and that the cell body is invaded immediately afterwards.

Diamond *et al.* (1956) concluded that after a mechanical stimulus to a Pacinian corpuscle, the all-or-nothing impulse was initiated at the first node of Ranvier. Their conclusion was based on experiments in which constant currents were passed between two pools divided by a petroleum jelly barrier which surrounded the nerve fibre between the second and third nodes of Ranvier (Figs. 2 and 18). This procedure caused currents, which decreased with distance from the barrier, to enter or leave the fibre at the nodes of Ranvier. When the pool containing the receptor was made positive, the membrane potential at the second node, i.e. the one nearest to the barrier on the receptor side, was raised and that across the first node was also raised but to a smaller

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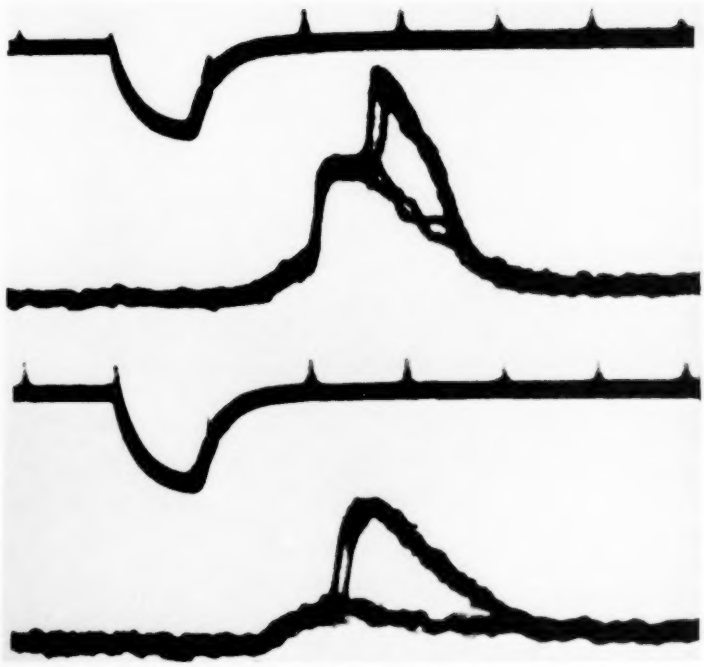

Fig. 15. Electrical activity of different parts of the nerve fibre of a Pacinian corpuscle. Top beams indicate amplitude and time course of stimulating displacement and time msec; bottom beams, records of fibre potentials. Both records consist of a number of superimposed traces. *a*, shows gentle slope of receptor potential then abrupt rise due to impulse at node 1, then, in some traces only, activity of node 2 (obtained with partial block by anodal polarization of receptor). *b*, increased anodal polarization; activity of node 2 has ceased and a small adjustment of stimulus amplitude has resulted in the critical condition for excitation of node 1 by the receptor potential (from DIAMOND *et al.*, 1956, Fig. 4).

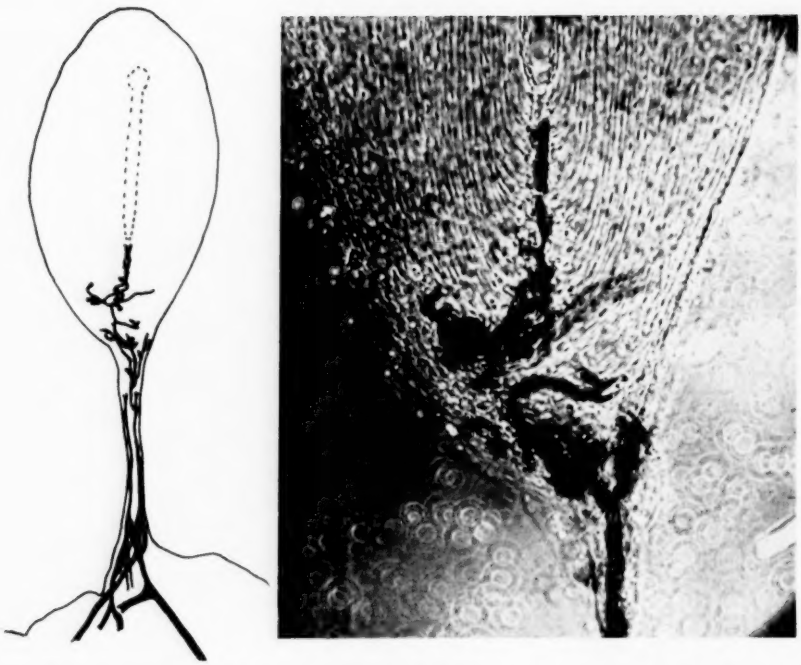

Fig. 16. Perfusion of a Pacinian corpuscle. Right, phase-contrast photomicrograph of a corpuscle injected with Indian ink. Left, drawing of vascular bed using all focal planes (from DIAMOND *et al.*, 1958b, Plate 1).

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extent. This increase in the membrane potential raised the threshold of the node and if it was raised enough that node remained inactive. Increasing the strength of the current, applied across the barrier, first divided the impulse, recorded across the same barrier, into two steps and a further increase blocked the second of these altogether (Fig. 15a). The second step was due to the activity of node 2 and the first to activity at node 1; this step can be seen rising from the graded receptor potential in Fig. 15*b*. Even the largest currents failed to produce any discontinuity in the first sharp step although the threshold for this part of the impulse was raised up to 7·5 times. This change of threshold indicated that the currents used should have been quite adequate to reveal an all-or-nothing event in the non-myelinated terminal if it existed.

There is also evidence that antidromically conducted impulses do not invade the terminations of the nerve fibres in these receptors. EYZAGUIRRE and KUFFLER (1955b) showed that the receptor potential of the stretch receptor of the crayfish was not abolished by an antidromically conducted nerve impulse. The receptor potential of this type of receptor is maintained as long as the receptor muscle is stretched; if the antidromic impulse had invaded that region of membrane in which the receptor potential was being generated, the low membrane impedance, associated with the passage of an impulse, would have been expected to short-circuit the terminal membrane and so wipe out the receptor potential for a time. DIAMOND *et al.* (1958a), using the Pacinian corpuscle, concluded that an antidromically conducted impulse does not invade the non-myelinated terminal. It has been stated above (p. 301) that an impulse can cause depression of a subsequent receptor potential, though this effect is less than that produced by a conditioning receptor potential. The depression caused by an antidromically conducted impulse was always slightly less than that caused by a mechanically excited impulse. If it is accepted that a mechanically excited all-or-nothing impulse is initiated at the first node of Ranvier then it must be concluded that the antidromically conducted impulse does not invade the terminal. If it did, it would cause a greater change of potential across the terminal membrane than that caused by the mechanically excited impulse, and it would also set up active processes in this membrane; in consequence an antidromically conducted impulse would be expected to cause more depression than a mechanically excited one, an expectation which is the reverse of the experimental finding.

There is thus evidence that in two types of mechanoreceptor all-or-nothing nerve impulses, whether due to excitation of the receptor or antidromically conducted, do not occur in the termination of the nerve fibre. These results are perhaps most easily explained by assuming that the terminal membrane is specialized and has not the mechanism

for propagating an all-or-nothing impulse; other explanations are, however, possible. The failure of a mechanical stimulus to produce an impulse in the terminals could be due to some degree of short-circuiting by the receptor activity; the antidromic impulses may have been blocked because of the relatively great attenuation, which probably occurs between the first node and the terminal, of the passive spread of membrane potential. Also it must be remembered that it is all-or-nothing activity that has been found lacking; graded activity associated with the same membrane mechanism has not been excluded.

If it is assumed that this region cannot conduct impulses, it can be argued that there is a specialization of the membrane of these terminals for the purpose of generating receptor potentials. This may well be so, but this evidence cannot be taken as proof that these membranes are activated by mechanical stimuli. MURRAY (1956) has argued that impulses are initiated away from the nerve terminals during mechanical excitation of the lateral line organs of *Xenopus laevis*. His reasoning is based on two observations: that the frequency of the impulses leaving the receptor is increased if a current is passed between two electrodes on the nerve, the cathode being placed close to the end-organ; and that the frequency is increased if the electrodes are placed across the skin with the anode on the outside. Observations confirming one or other part of this experiment have been made on a number of other preparations: toad skin (MARUHASHI *et al.*, 1952), the labyrinth of the thornback ray (LÖWENSTEIN, 1955) and the lateral line organs of the Japanese eel (KATSUKI and YOSHINO, 1952). There are reasons for believing that the increases in frequency are not due to excitation at a separate site on the nerve fibre; it can therefore reasonably be assumed that the current, by leaving the fibre at the site at which impulses are initiated, summates with that due to the receptor potential. It is difficult to see how this can happen except in the way suggested by MURRAY, that is by assuming that the impulses are initiated away from the terminal. The majority of these preparations have receptors associated with non-nervous sense cells, i.e. hair cells, and there is evidence that the hair cells and not the nerve terminals generate the microphonic potentials. Nothing is known about antidromic invasion of the terminations of these nerve fibres and it may be that the site of impulse initiation depends solely on the distribution of the exciting current and not on any special properties of the membrane.

2.2*c*. *Chemical excitation.* There is another way in which mechanoreceptors, and other receptors, differ from most parts of nerve cells. A number of receptors have been shown to be excited by acetylcholine and similar substances. This action, which is probably a direct depolarization of some part of the ending, is prevented by blocking agents; however, neither blocking agents nor anticholinesterases have

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any effect on the physiological behaviour of these mechanoreceptors. Adrenaline can also modify receptor behaviour. This subject will not be considered in detail here, since it has been reviewed recently by GRAY and DIAMOND (1957). It is not known precisely where these substances act but it is known that the drug effects summate directly with the receptor activity. It is possible that the regions excited by acetylcholine are also those which do not conduct impulses, which generate receptor potentials and which have no Schwann cell covering.

2.3. *Source of energy.* There are many mechanoreceptors which signal a particular mechanical state by a particular frequency of impulse discharge. Such receptors have been observed to maintain a constant frequency for hours. It may be assumed that these impulses are initiated by receptor potentials; this would indicate that receptor potentials may last for hours, though actual recordings which have been published do not exceed 10 sec. Nerve cell membranes have resistances of the order 1000 Ω/cm^2 (squid nerve fibre 700 Ω/cm^2, lobster nerve fibre 2000 Ω/cm^2, crab nerve fibre 5000 Ω/cm^2 (KATZ, 1952), mammalian motor neurone 500 Ω/cm^2 (COOMBS *et al.*, 1955a)), and these prolonged receptor potentials must, therefore, cause current to flow continuously through neighbouring parts of the cell membrane (Fig. 18). This current flowing along these potential gradients represents a continuous dissipation of energy. This clearly cannot be derived from the work done during the initial change of state. This argument applies to the receptor potential of the crayfish stretch receptor and the static phase of that of the frog's muscle spindle; it does not apply to the dynamic phase of the spindle potential or to the receptor potential of the cat's Pacinian corpuscle.

If the energy for the electrical output does not derive from the external work it must come from an internal source. A source of energy is in fact available across the membrane of the terminal portions of the nerve fibre, the site at which it is likely that the receptor potentials are generated. In the introduction it has already been pointed out that there is a resting potential across the membrane of a nerve fibre. This potential can be accounted for by differences in the permeabilities of the membrane to the principal ions. The external ions are almost entirely Na^+ and Cl^-, and the resting cell membrane while being relatively permeable to the latter is much less permeable to Na^+. The internal ions are predominantly K^+, to which the membrane is relatively permeable, and large anions, which do not easily pass through. The cell maintains this state by actively extruding Na^+. As long as the membrane remains much more permeable to K^+ and Cl^- than to other ions the membrane potential is given by $E = RT/F \log_n (K_1/K_0)$, where K_1 and K_0 are the concentrations of K^+ inside and outside the fibre respectively; R = gas constant, T = absolute temperature and

F = Faraday constant (HODGKIN, 1951). In the healthy state resting potentials may be as high as 80–90 mV. Under many experimental conditions, however, membrane potentials are lower and the simple equation just quoted does not necessarily apply. While energy is stored in this biological battery, it must be remembered that the energy is derived from certain chemical reactions which do work in extruding Na^+ against its electrochemical gradient.

2.4. *Ionic movements associated with receptor potentials.* There are reasons to suppose that receptor potentials are generated across the membrane of the nerve terminals; there are reasons to suppose that this membrane may be specialized in a number of ways; an internal source of energy is required and there is one available at this site. This suggests that receptor potentials result from some change in the membrane that will allow this energy to be utilized.

2.4*a*. *The capacitance hypothesis.* KATZ (1950b) discussed a suggestion that a change in membrane capacity might account for the dynamic phase of the spindle receptor potential; an increase in capacity might be expected if the membrane were stretched, thus becoming greater in area and thinner; the consequent redistribution of charge would cause a fall in membrane potential. KATZ concluded on quantitative grounds that this explanation was unlikely unless a degree of mechanical amplification occurred in the system. In the Pacinian corpuscle, the mechanism seems unlikely as the large increases in area that are demanded are unlikely to occur. On this hypothesis, the size of the receptor potential would not be changed by changing the ionic content of the surrounding medium, provided this did not cause a change in membrane potential. It will be shown below (p. 314) that the receptor potential is related in a graded manner to the Na^+ concentration; changes in the concentration of this ion would not be expected to cause any change in membrane potential.

2.4*b*. *Ionic movements.* The most likely explanation of the receptor potential is that ions transport charge across the membrane. The polarity of the change means that either external cations must enter the fibre or internal anions must leave. The internal anions of nerve fibres are mostly organic in nature and relatively large; they are not known to play any important part in potential changes across cell membranes at other sites. The principal external cation is Na^+; its concentration in the external fluid greatly exceeds that of any other cation and its electrochemical gradient is large and directed inwards. Na^+ is known to play a major role in certain biological potentials. If Na^+ transports part of the charge required for a receptor potential, changes in the concentration of Na^+ in the external fluid would be expected to modify both the rate of rise and the amplitude of the receptor potential, and these changes should be related in a graded

manner to the Na^+ concentration. KATZ (1950b) using the frog's muscle spindle and GRAY and SATO (1953) using the Pacinian corpuscle tried bathing these preparations in Na^+ free solutions, but in neither instance was any effect on the receptor potential observed. The times of soaking in Na^+ free solutions required to abolish impulses were very similar in the two preparations; this was also true of the concentrations of procaine required to produce comparable effects. These facts suggest that the rates of movement of these substances are similar in

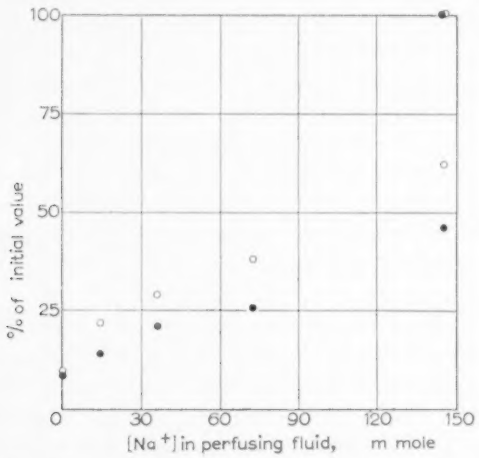

Fig. 17. Effects of Na^+ concentration on the receptor potentials of a Pacinian corpuscle. Abscissa, sodium concentration (millimoles); ordinate, amplitude (●) and rate of rise (○) of receptor potentials as percentage of initial value. Points obtained in following order: high value with full Na, no Na, 10 per cent of normal Na, 25 per cent Na, 50 per cent Na, low value with full Na (from DIAMOND *et al.*, 1958b Fig. 8).

the two systems. GRAY and SATO (1955), using labelled ions, showed that the movement of Na^+, K^+ and Br^- through the lamellae of the Pacinian corpuscle was very slow and it seems certain that the failure to demonstrate an effect of Na^+ free solutions on receptor potentials was due to this slow rate of ionic movement.

2.4*c*. *Perfusion of Pacinian corpuscles.* DIAMOND *et al.* (1958b) perfused Pacinian corpuscles through their circulation. Capillaries are found in the central pole of these bodies, and a single capillary loop is normally seen to run to the point at which the nerve fibre enters the central core (Fig. 16); this is the point at which the fibre loses its myelin and Schwann cell. No capillaries are found in the central core and only rarely peripheral to this point in any part of the interior or the organ (SCHUMAKER, 1911; SHEEHAN, 1933; PEASE and QUILLIAM, 1957). Perfusion of this capillary system with Na^+ free solutions (made

isotonic with choline chloride or sucrose) caused impulses to be abolished in as little as 1·5 min; receptor potentials became very small, and a constant minimum value was reached in 10–20 min. These times lend support to the conclusion already discussed (p. 308) that the receptor potential is generated in the non-myelinated terminal while impulses are initiated at the first node of Ranvier. It looks as if a comparable situation may occur in the muscle spindle; for KATZ (1950b) found that he could abolish all impulse activity by bathing the receptor in Na^+ free solution, but he was unable to affect the receptor potential. In the absence of Na^+ the rate of rise and amplitude of the receptor potential of the Pacinian corpuscle fell to a fraction of their normal value and both these parameters were related in a graded manner to the Na^+ concentration; this is illustrated in Fig. 17 in which this grading can be seen in spite of the decline, which occurred during the prolonged perfusion, of the size and rate of rise of the potential. The receptor potential was not abolished, a small part, perhaps 10 per cent, remaining. It is likely that this residue represents a contribution to the membrane current by other ions, but this cannot be stated with certainty.

2.4*d*. *Stimulus–response relationships and membrane permeability.* If ions transport charge across the membrane, the movement must result from either a change in the driving force or a change in the resistance to the movement of one or more ions provided by the membrane. Since it is the cause of a change in potential that is being discussed, a change in the driving force must mean a change in ionic concentrations; it is difficult to see how this could be effected. A change in permeability seems probable and such a hypothesis is consistent with the relationships that have been observed between the amplitude and rate of rise of the receptor potential of the Pacinian corpuscle and the amplitude of the stimulating displacement. If, for example, the membrane became more permeable than normal, in a non-specific manner, to ions of a greater volume, the relative permeabilities of the principal ions would approach each other and the total permeability would increase. The limiting amplitude of a receptor potential would approach the value of the resting potential; that is to say that, at the peak of a receptor potential, the membrane potential would approach zero. A theoretical approach to the general problem has been made by GOLDMAN (1943) and HODGKIN and KATZ (1949). If it is assumed that there is a constant potential field throughout the membrane and that there is no net membrane current at the time in question, then the membrane potential should be given by:

$$RT/F \,.\, \log_n \left\{ \frac{P_k K_i + P_n Na_i + P_c Cl_o}{P_k K_o + P_n Na_o + P_c Cl_i} \right\}$$

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where P_k, P_n and P_c are permeability coefficients for K^+, Na^+ and Cl^- respectively. K_i, Na_i and Cl_i are concentrations inside the fibre and K_0, Na_0 and Cl_0 are concentrations outside the fibre. This equation derived by HODGKIN and KATZ (1949) refers only to the principal monovalent ions. Inclusion of the divalent cations can be shown, by an extension of the equation, to make little difference to the results. If this relation represents the situation, then the maximum receptor potential will depend on the ratios of the permeabilities, while the final membrane conductance will depend on the actual values. Limiting maxima are known in two comparable situations; the membrane potential at the peak of a nerve impulse is limited by the Na^+ equilibrium potential since P_n becomes much greater than P_k and P_c in which case the above equation reduces to:

$$RT/F \,.\, \log_n \{Na_i/Na_0\}$$

Skeletal muscle end-plate potentials (FATT and KATZ, 1951) and the excitatory synaptic potentials of the cat's motor neurone (COOMBS *et al.*, 1955b) reach, at their maxima, values of membrane potential that are within a few mV of zero. There is reason to suppose that these particular changes of potential are caused by a non-specific increase in the permeability to many ions; this increase, while giving rise to a general increase in membrane conductance equalizes the values of the permeability coefficients. If the genesis of the receptor potential is similar, the relation found between receptor potential amplitude and the mechanical displacement and also the order of magnitude observed for the maximum value of a receptor potential would be expected.

The rate of rise of the receptor potential continues to increase with increasing stimulus strength at values at which the amplitude of the receptor potential is maximal (Fig. 8*a*). The rate of rise of the potential will depend on the rate at which the membrane capacity is being discharged (i.e. on the membrane current). This finding is to be expected on this hypothesis, since membrane current will depend on the total conductance and will not be limited by those factors that would limit the amplitude of a receptor potential.

2.4*e*. *Depression and membrane permeability.* The phenomenon of depression which has been found in the Pacinian corpuscle needs to be considered in the light of this hypothesis. There is evidence that all-or-nothing nerve impulses, whether set up by mechanical excitation of the receptor or conducted antidromically into the ending, do not occur in the non-myelinated terminal of this end-organ. Impulses can, however, cause some degree of depression of a subsequent receptor potential (p. 301). Impulse activity at the first node of Ranvier will cause current to pass outwards across the membrane of the

non-myelinated terminal and this will cause a fall of membrane potential in this region. It seems that the depression resulting from an impulse can only be due to such a change in membrane potential. A reduction in the potential field might reduce the degree of molecular orientation in the membrane and it is possible that such orientation is a prerequisite for activation by mechanical means. The change of field does not, however, appear to be an adequate cause of activity in itself; this conclusion is reached because the change in field produced by an antidromic impulse does not appear to cause activity in the non-myelinated terminal. Receptor potentials cause appreciably more depression; in this instance, however, the terminal is itself active and the current flow is inward. It is possible that it is the change of potential *per se* that causes the depression that follows a receptor potential, but this seems on the present evidence to be an incomplete explanation (see p. 302). There is a relationship between depression and rate of rise of receptor potential (Diamond *et al.*, 1958a), and this is what would be expected if the processes underlying a change in membrane permeability were temporarily inactivated by processes underlying activity.

2.4*f*. *Other receptors.* The evidence discussed in this section has been almost entirely derived from the Pacinian corpuscle. The question of how far such a hypothesis would fit the results obtained from the other receptors, now arises. There is little relevant information to discuss at present, but reference must be made to the differences between the dynamic and static phases of the receptor potential of the frog's muscle spindle that have been discussed by Katz (1950b). The different behaviour of the two phases to procaine and a distinct discontinuity between them led Katz to consider the possibility of separate mechanisms. It might well be possible to explain the two phases in terms of the two processes known to occur in the Pacinian corpuscle, that of excitation and that of depression; until we have information about the time courses of these two processes in the biological transducer itself this suggestion must, however, remain speculative.

2.5. *Theories of mechanical action.* Even if it is assumed that the electrical output of the transducer is caused by a change in the permeability of the nerve fibre membrane, we are still left with the problem of how the mechanical events bring about such a change. There is at present no positive evidence as to the nature of this process, but certain suggestions can be considered in relation to a few relevant experimental findings. The three suggestions considered in this section are not, of course, the only possible explanations; they do, however, happen to be related to particular investigations and are mentioned for this reason.

2.5*a*. *Chemical transmitters.* Those biological potentials that show the greatest resemblance to receptor potentials are those found at

synapses and at end-plates. In these instances there is good evidence that a change of membrane permeability is brought about by the action of a chemical agent, which is released from the pre-junctional nerve fibre (FATT, 1954). In certain instances this substance is known to be acetylcholine. Acetylcholine has been suggested as an intermediary in the excitation of receptors on several occasions (v. EULER *et al.*, 1941; LANDGREN *et al.*, 1954; KUIPER, 1956). The work in this field has been done on receptors other than those discussed in this article and the bulk of the evidence is pharmacological. The subject has been reviewed recently by GRAY and DIAMOND (1957) and will not be considered again here; the reviewers conclude that the evidence is definitely against any participation of acetylcholine in the process of excitation of mechanoreceptors.

One of the difficulties facing any theory of chemical transmission is that it is difficult in many receptors to identify any structure that could release the transmitter. This, of course, does not apply to end-organs with "sense cells"; nor does it apply to the Pacinian corpuscle, in which the cells of the central core could act in such a way. This central core does in fact contain a high concentration of pseudo-cholinesterase (HEBB and HILL, 1955; BECKETT *et al.*, 1956), which enzyme, while similar in its behaviour to true cholinesterase, is not found at junctions at which acetylcholine is a transmitter; the destruction of both types of cholinesterase does not affect the sensitivity of the ending (DIAMOND and GRAY, unpublished). Furthermore, acetylcholine is not found in Pacinian corpuscles (BROWN and GRAY, 1948). Another more general point which suggests that the processes in the Pacinian corpuscle differ from those at mammalian end-plates and synapses is that the latency is shorter. The latency between the beginning of the stimulating displacement and the beginning of the first sign of the receptor potential was found by GRAY and SATO (1953) to be usually less than 0·2 msec for preparations at room temperature; on the other hand the fastest synaptic delay found in the mammal at body temperature is *c.* 0·5 msec; in general synaptic delays are shorter at junctions between fibres that conduct impulses rapidly, but the fibre from the Pacinian corpuscle is one which conducts relatively slowly.

2.5*b*. *Membrane area.* A simple explanation of the mechanical action might be that it stretches the fibre membrane. The only displacement that has been detected in the region of the central core of the Pacinian corpuscle is in a radial direction and this displacement occurs over the whole length of the core (p. 303). HUBBARD (1958) therefore calculated values of the ratio $\Delta A/A : \Delta b/b$ for different values of a/b (where A = membrane area, a = major semi-axis of the elliptical cross-section of the nerve fibre and b = minor semi-axis); the calculations were based on the assumptions that the nerve fibre volume is constant and

that Δb is small compared with b. Clearly when $a/b = 1$, i.e. a fibre of circular cross-section, the surface area will not change with relatively small changes in radius ($dA/db = 0$). With increasing eccentricity of the fibre cross-section $\Delta A/A$ increases rapidly for a given value of $\Delta b/b$. The ratio a/b for the non-myelinated terminal of the Pacinian corpuscle is about 1·5 and the corresponding value of the ratio $\Delta A/A : \Delta b/b$ is approximately 0·3. This means that, because the nerve fibre in this situation is unlike other fibres, elliptical, it is possible for a radial displacement to cause a change in membrane area; it does not of course follow that this is an essential part of the transducer mechanism.

2.5*c*. *Pressure changes.* Another suggestion that has been put forward by D. E. GOLDMAN is that, associated with the observed displacements (p. 303), there is a change of pressure and that this change of pressure causes certain changes in the molecular organization either of the membrane itself or of the hydrated ions on either side of it. If this were so, it should be possible to excite a mechanoreceptor with a pressure-pulse, arranged in such a way that no distortions other than those necessitated by the compressibility of water occurred. In preliminary experiments on Pacinian corpuscles and frog's skin (GOLDMAN and GRAY, unpublished) excitation only occurred when distortion could occur; it is not yet known what pressures might be developed in a corpuscle, or at any other receptor, when using displacements as stimuli and there is considerable uncertainty as to the pressures developed during the experiments just quoted; a definite conclusion must therefore be left till further experiments have been done.

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3. *Transmission of the receptor potential*

All the experimental evidence regarding the receptor potential that has been quoted refers, not to the direct output of the transducer, but to an electrical change recorded at a distance; the potential change is transmitted because the fibre acts as an electric cable. Any electrical distortion occurring in this cable handicaps the investigation of the transducer mechanism itself. However, the problem is not entirely an experimental one, because nerve impulses are initiated away from the regions in which the receptor potentials are generated.

In the Pacinian corpuscle the structural situation is fairly clear (Fig. 18). The receptor potential appears to be generated in the terminal (T), and the impulse to be initiated at the first node ($N1$); records have been obtained by recording current flow across a deliberately increased external resistance between the second and third nodes of Ranvier ($N2$ and $N3$) (DIAMOND *et al.*, 1956). An equivalent circuit, which is probably a reasonable representation of the situation, is also given in Fig. 18. The resistance of the external fluid

is ignored, except for the recording resistance, and the inside of the fibre is represented as a series of resistances each equivalent to the length of an internode. Nodes are represented as capacities in parallel with resistances and a distributed capacity is indicated across the myelin. The terminal is represented as a generator, with its internal resistance, in parallel with a capacity and a resistance. The initiation of impulses depends on the peak potential appearing across the capacity of node 1, and the recorded potential is related to the current through

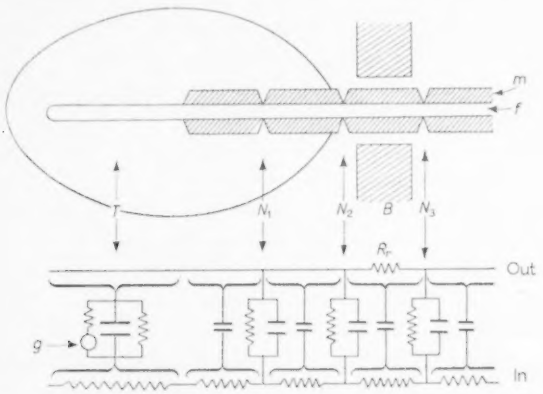

Fig. 18. Diagram to illustrate electrical properties of the Pacinian corpuscle. *f*, nerve fibre; *m*, myelin; *T*, non-myelinated terminal; *N*1, *N*2, *N*3, first, second, third nodes of Ranvier; *B*, barrier; R_r, recording resistance; *g*, current generator. Brackets indicate distributed properties.

the recording resistance (R_r); these quantities need to be related to the intensity and time course of the current from the generator. The dimensions of the fibre are known and certain physical constants can be assumed: the resistivity of the inside of the fibre, the dielectric constant of myelin and the resistivity of myelin (this is very high and has been ignored in Fig. 18). The resistance and capacitance of the nodes is not known and they may differ significantly from the values given by TASAKI (1955) for certain frog fibres. The properties of the terminal membrane are also unknown though it is possible that the membrane capacity is similar to those which have been measured in other nerve cells, i.e. about 1–6 μF/cm^2 (see KATZ, 1952; ECCLES, 1957). More information is therefore required before it is possible to determine the intensity and time course of the current from the generator.

VI. CONCLUSIONS

In the mechanoreceptors which have been considered it is probable that the receptor potential is generated across the membrane of the nerve

terminal. In one way or another these terminals are all specialized and it is possible that there is a causal association between the absence of the Schwann cell sheath, the ability to generate receptor potentials and the absence of nerve impulses from these terminals. Certainly in two and probably in all three receptors an internal source of energy is necessary and this is available in the form of ionic concentration gradients across the nerve membrane. The amplitude and rate of rise of the receptor potential of the Pacinian corpuscle are related in a graded manner to the concentration of Na^+ in the external fluid, and this and other experimental observations are consistent with the hypothesis that Na^+, and probably other ions as well, move down their electrochemical gradients because of a change in the permeability of the membrane. There may be considerable loss and also modification of the mechanical energy in the course of its transmission to the active structures of the receptor. The output of the receptor is separated both from the site of recording and from the site of impulse initiation by a conducting system whose impedance introduces an unknown amount of distortion.

Throughout this article it has been implicit in the argument that the three receptors function in the same manner. Where the evidence has been derived from more than one type, agreement has always been reasonable. The one piece of evidence which suggests that there might be more than one mechanism, is that there are certain differences between the two phases of the receptor potential of the frog's muscle spindle. This has already been discussed, and a suggestion made as to how these results can be fitted into the single scheme presented here (p. 316). However, there is no proof that the mechanisms of these three receptors are the same and an effort should be made to obtain complete evidence in each individual case.

Finally a word of speculation about the relation of the receptor potentials discussed in this article to the activity of complex mechanoreceptor organs which contain hair cells. The hair cells appear to be responsible for the microphonic potentials and it is widely assumed that these potentials are directly or indirectly responsible for the initiation of impulses in the associated nerve terminals. These terminals are structurally similar to the nerve terminals which are discussed in this article and which appear to generate receptor potentials; furthermore nerve impulses are initiated away from the terminations of the nerve fibre in both situations. It would be very interesting to know whether any graded potential change was actively developed across the membranes of nerve terminals associated with hair cells and if so whether or not such a change could be produced by a mechanical deformation. The finding of such potential changes would raise an interesting point in relation to the phylogenetic development of the

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more specialized mechanoreceptors. Is it possible that hair cells and the current fields that they produce have been superimposed on mechanoreceptors of a more primitive type with the result that the sensitivity has been increased to the extreme levels found in some of these organs?

It will be obvious to anyone who has read through this article that there are many gaps small and large in the argument. I have included a number of topics on which the evidence is at present very slight and others in which the lack of certain quantitative values has made such information as we have of little use. This has been done because I felt it to be of value to collect together as much evidence as possible and so show more clearly where the gaps in our knowledge lie.

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GRADIENT CENTRIFUGATION OF CELL PARTICLES THEORY AND APPLICATIONS

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GRADIENT CENTRIFUGATION OF CELL PARTICLES: THEORY AND APPLICATIONS

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I. Glossary of Main Symbols

B	defined by equation (23).
C	particle concentration.
C_i	initial particle concentration.
C_s	concentration of particles having a sedimentation constant smaller than or equal to s.
C_{si}	initial concentration of particles having a sedimentation constant smaller than or equal to s.
c	molar concentration of solutes.
$F(s)$	frequency function of sedimentation constants (equation 36).
f	frictional coefficient.
f_o	frictional coefficient of spherical particle (equation 3).
k	defined by equation (20).
k_s	value of k in 0·265 molal sucrose.
m	molal concentration of solutes.
m_e	molal concentration of solutes in medium isopycnic with particles.
r_o	radius of spherical particle.
S	Svedberg unit $= 10^{-13}$ sec.
s	sedimentation constant.
s_s	standard sedimentation constant (i.e. in 0·265 molal aqueous sucrose at 0°) (equation 21).
t	time.
$u(x)$	function defined by equation (40).
V	average partial molal volume of solutes.
v	sedimentation velocity.
W	time-integral of squared angular velocity (equation 29).
x	radial distance of particle.
x_b	radial distance of boundary.
x_f	radial distance of bottom of tube.
x_i	initial radial distance of particle.
x_o	radial distance of meniscus.
α	amount of osmotically active substances inside the particle (p. 333).
β	ratio of ϕ_m to ϕ_d.
γ	geometrical factor (pp. 330, 341).

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η viscosity of medium.
η_s viscosity of 0·265 molal sucrose at 0° ($2{\cdot}28 \times 10^{-2}$).
θ frictional ratio (equation 5).
μ mass of particle.
μ_d mass of osmotically dehydrated particle.
ρ_d density of osmotically dehydrated particle.
ρ_e equilibrium or isopycnic density.
ρ_{es} equilibrium density in aqueous sucrose solution.
ρ_m density of medium.
ρ_{ms} density of 0·265 molal sucrose at 0° (1·0341).
ρ_p density of particle.
ρ_u density of solutes in solution.
ρ_w density of solvent.
$\Phi(x)$ function defined by equation (42).
ϕ volume of particle.
ϕ_d volume of osmotically dehydrated particle.
ϕ_m compartment accessible to external solutes.
ϕ' apparent volume of particle (equation 17).
ϕ'_s value of ϕ' in 0·265 molal sucrose.
$\Psi(x)$ function defined by equation (43).
ω angular velocity.

II. Introduction

Density gradients have been used in centrifugation with two distinct aims. One technique, which is referred to by Anderson (1955a) as "*isopycnic gradient centrifugation*", makes use of fairly steep gradients extending over the range of density of the particles to be separated; centrifugation is pursued until each particle has reached a position corresponding to its own density. In the other, termed "*gradient differential centrifugation*" by Anderson (1955a), a fairly small gradient is used to stabilize the tube contents against convection currents and other anomalies; this permits the operation to be interrupted before complete sedimentation and the level reached by each group of particles to be determined by suitable methods. In the latter technique separation is due to differences in sedimentation rate whereas in the former it is theoretically dependent only on differences in density.

Both methods make use of preformed gradients which are not appreciably disturbed by centrifugation. They differ in this respect from the technique recently described by Meselson *et al.* (1957), in which a fairly dense solute (CsCl) is used and the gradient is established during operation under the opposed influences of the centrifugal force and of diffusion. In view of the time necessary to accomplish this, it is doubtful that this elegant method will be applicable to fragile subcellular particles.

BEHRENS (1938), who used non-aqueous gradients, must be credited with having first put into practice the isopycnic centrifugal method for density measurements on biological components and later for separation purposes. More recently, sucrose gradients with or without addition of other components, have been applied to the fractionation of virus or cell material by BRAKKE (1951, 1953), HOLTER *et al.* (1953), SCHNEIDER *et al.* (1953), KUFF and SCHNEIDER (1954), OTTESEN and WEBER (1955), WEBER (1955), BLASCHKO *et al.* (1957) and BARNETT *et al.* (1958).

Stabilizing gradients were first used by PICKELS (1943) with the angle centrifuge and by KAHLER and LLOYD (1951) with a horizontal rotor of their own construction. The technique has since been perfected and applied extensively by KUFF and co-workers (HOGEBOOM and KUFF, 1954; KUFF *et al.*, 1955; LEVINTOW *et al.*, 1955; KUFF *et al.*, 1956), THOMSON and his group (THOMSON and MIKUTA, 1954; THOMSON and MOSS, 1956; THOMSON and KLIPFEL, 1957, 1958), ANDERSON (1955a, 1956) and ALBRIGHT and ANDERSON (1958).

Both methods are also used in this laboratory, as they are known to be in many others, and there is little doubt that they will tend to complete and even replace in many cases the classical technique of differential centrifugation, at least when the latter is used as an analytical tool. They involve, however, a number of theoretical and practical difficulties, which have been dealt with partly in some of the papers mentioned above, but have so far not been treated fully in all their aspects. It is the aim of the present paper to provide workers interested in the use of these methods with a compendium summarizing the necessary information. It includes a number of original developments, which to our knowledge have not been published elsewhere.

Gradient centrifugation is of course only a special application of the centrifugal technique, complicated by the existence of density, viscosity and osmotic pressure gradients within the tube. Standard works on centrifugation such as those of SVEDBERG and PEDERSEN (1940), NICHOLS and BAILEY (1949), AMBLER and KEITH (1956) and OGSTON (1956) will therefore serve as a useful introduction to some of the present discussions. The reader is also referred to previous recent reviews (DE DUVE and BERTHET, 1954; HOGEBOOM and SCHNEIDER, 1955; ANDERSON, 1956; HOGEBOOM *et al.*, 1957) for a critical survey of the results achieved by the conventional methods of differential centrifugation.

In the following treatment, it will be assumed that biological material has been centrifuged in a tube containing a given concentration gradient of sucrose or of some other substance and that the distribution of the material is then determined within the tube and expressed as a function of the radial distance from the axis during

centrifugation. In the first part, the factors affecting the distribution of the sedimenting particles are studied and a general equation is worked out, applicable to all conditions. The second part deals with the mathematical analysis of the observed distribution curves. The practical procedures used for preparing the gradients, ensuring smooth centrifugation conditions and sampling the tubes are described in the third part.

III. Factors Affecting the Distribution of Sedimenting Particles

Under ideal conditions, the distribution of sedimenting particles after spinning for a given length of time at a given speed depends on the geometry of the centrifugal system, the conditions within the tube at zero time, the rate of sedimentation of the particles and their rate of diffusion. Under non-ideal conditions, convective disturbances must also be taken into account.

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1. *Geometry of the centrifugal system*

1.1. *Parallel sedimentation in cylindrical tubes.* If sedimentation occurs everywhere along a direction parallel with the walls of the tube, the geometry of the system by itself will exert no influence on the particle distribution. This condition is approached in simple gravity sedimentation and possibly, as will be shown below, in some types of centrifugal sedimentation.

1.2. *Radial sedimentation in sector-shaped tubes.* The cross-sectional area of a sector increases linearly with the distance from the centre (axis of rotation). Therefore, irrespective of all other factors which influence the particle distribution, the geometry of the system imposes a progressive dilution on the sedimenting particles, in the ratio x_i/x, x_i and x being the initial and final positions of the particles, measured radially from the axis of rotation.

1.3. *Radial sedimentation in horizontal cylindrical tubes.* Except in one case of low-speed centrifugation (Anderson, 1955a), ideal sector-shaped tubes have not been used with preparative equipment, but horizontal swinging-bucket rotors accommodating cylindrical tubes are now available which will stand angular velocities of up to 39,000 rev/min. In such a system, the particle concentration within the main part of the fluid will suffer a progressive decrease owing to the radial direction of sedimentation, while crescents of concentrated particles will tend to form along the sides of the tube. These crescents will continue to move downwards subject to the tangential component of the centrifugal field, which in the usual systems, characterized by a fairly small ratio of tube-section to radial distance, represents more than 99 per cent of the total field. If the walls of the tube are wetted by the suspension

medium and offer about the same frictional resistance as the medium itself, the rate of sedimentation of these crescents will depend on their state of aggregation and on the convection currents they may tend to produce. The latter should be minimal in a density gradient and one may therefore consider two simple possibilities: (*a*) rapid sedimentation of the crescents as units of aggregated particles; (*b*) sedimentation of the particles in the crescents as discrete units at approximately the same rate as those moving freely within the fluid.

In case (*a*), all particles hitting the side-walls are effectively removed by rapid sedimentation to the bottom; therefore, the particle distribution within the supernatant will be affected by the same geometrical factor as in true radial sedimentation, i.e. the particle concentration within the fluid will be the same as if centrifugation had been performed in a sector-shaped tube. In case (*b*), the *average* concentration of the particles at a given level of the tube will be the same as in ideal parallel sedimentation, and no geometrical correction will have to be applied. According to HOGEBOOM and KUFF (1954) and KUFF *et al.* (1956), the results obtained both with soluble proteins and with cell particulates afford greater support to the latter than to the former possibility. THOMSON and co-workers (1954, 1956, 1957), on the other hand, are in favour of immediate sedimentation after side-wall impaction but actually apply a correction amounting only to about one-half the theoretical correction and which they have found to be empirically satisfactory (personal communication). It is possible, therefore, that the true situation is intermediate between the two extremes discussed above. Until more information on this point becomes available, it will be convenient to consider the geometrical dilution factor in horizontal cylindrical tubes as being represented by $(x_i/x)^\gamma$, γ having a value between 0 (parallel sedimentation) and 1 (radial sedimentation). A more rigorous justification of this expression will be given later (see p. 341).

1.4. *Radial sedimentation in cylindrical tubes kept at a fixed angle with respect to the rotation axis.* Although angle-head centrifuges have been used successfully in semi-analytical work, they bring about considerably greater hindrances to normal sedimentation than do the horizontal heads. PICKELS (1943) has made an experimental and theoretical study of these hindrances and found that they tend to produce convection currents which favour sedimentation along the outer wall of the tube. As a first approximation, boundary movement and sediment accumulation proceed as they would in a horizontal cell of comparable radial dimensions. It seems likely, however, that the particle distribution within the tube will be severely affected by the convection currents and by the changes in fluid position occurring during acceleration and deceleration. That such is indeed the case is

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shown by the results of KAHLER and LLOYD (1951), who have compared the two types of rotors. Angle-heads do not therefore appear to be well suited for distribution studies and the following discussions will be restricted to horizontal heads.

2. *Initial conditions*

The initial conditions are defined by the distribution of the medium and of the particles at zero time. The medium may either be of uniform composition or contain one or more components the concentration of which increases continuously from the top to the bottom of the tube (concentration gradients). Discontinuous gradients have also been used, but they give rise to artifacts (ANDERSON, 1955a) and do not lend themselves to a simple mathematical treatment. They will therefore not be considered here. The particles may either be distributed homogeneously within the tube or be added as a separate layer deposited above or below the medium.

3. *Sedimentation rate*

3.1. *The rate equation.* The most general form of the Svedberg equation may be written as follows:

$$v = \frac{\mathrm{d}x}{\mathrm{d}t} = \frac{\phi(\rho_p - \rho_m)}{f}\,\omega^2 x \qquad \ldots.(1)$$

where v = the radial velocity of a sedimenting particle in cm sec^{-1},

ϕ = the volume of the particle, in cm^3,

ρ_p = the density of the particle, in g cm^{-3},

ρ_m = the density of the medium, in g cm^{-3},

f = the frictional coefficient, in g sec^{-1},

ω = the angular velocity of the centrifuge, in rad sec^{-1},

x = the radial distance from the axis of rotation, in cm.

The sedimentation constant s is defined as the velocity of sedimentation per unit centrifugal field:

$$s = \frac{v}{\omega^2 x} = \frac{\phi(\rho_p - \rho_m)}{f} \qquad \ldots.(2)$$

It is measured in seconds or in Svedberg units, 1 S being equal to 10^{-13} sec. Its value depends on the characteristics of the particle, on the composition of the medium, on the temperature and on the particle concentration. The influence on the terms in equation (2) of the increase in pressure brought about by the centrifugal field will not be discussed. It is negligible at the speeds generally used for the study of cell particles.

3.2. *Influence of particle characteristics.* According to Stokes' law, the frictional coefficient f_o of a spherical particle is given by:

$$f_o = 6\pi\eta r_o \qquad \ldots.(3)$$

in which η = the viscosity of the medium in poises (g cm^{-1} sec^{-1}),
r_o = the radius of the particle, in centimetres.

Thus, for a spherical particle, equation (2) may be written:

$$s = \frac{\phi(\rho_p - \rho_m)}{6\pi\eta r_o} = \frac{2r_o^2(\rho_p - \rho_m)}{9\eta} \qquad \ldots.(4)$$

This equation may still be applied to non-spherical particles, by introducing the shape factor or frictional ratio:

$$\theta = f/f_o \qquad \ldots.(5)$$

Then:

$$s = \frac{\phi(\rho_p - \rho_m)}{6\pi\eta r_o\theta} = \frac{2r_o^2(\rho_p - \rho_m)}{9\eta\theta} \qquad \ldots.(6)$$

where r_o is the radius of the sphere of volume equal to that of the particle and θ the ratio of the frictional coefficient of the particle to that of a spherical particle of equal volume.

Values of θ for ellipsoids of revolution have been tabulated by SVEDBERG and PEDERSEN (1940). A few representative values are given in Table 1, as a function of the half-axis of revolution, r_1, and of the equatorial radius r_2 of the ellipsoid.

TABLE 1

Frictional Ratio θ for Ellipsoids of Revolution (from SVEDBERG *and* PEDERSEN, 1940)

Prolate ($r_1 > r_2$)		*Oblate* ($r_2 > r_1$)	
r_1/r_2	$\theta = f/f_o$	r_2/r_1	$\theta = f/f_o$
1·0	1·000	1·0	1·000
2·0	1·044	2·0	1·042
3·0	1·112	3·0	1·105
4·0	1·182	4·0	1·165
5·0	1·255	5·0	1·224
6·0	1·314	6·0	1·277
7·0	1·375	7·0	1·326
8·0	1·433	8·0	1·374
9·0	1·490	9·0	1·416
10·0	1·543	10·0	1·458
20·0	1·996	20·0	1·782

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3.3. *Influence of medium composition.* Properties of the medium which enter explicitly into equation (6) are the density ρ_m and the viscosity η. However, in the case of cell particles behaving as osmotic systems, the volume, radius and density of the particles become themselves functions of the composition of the medium. The first attempt to correct for osmotic changes has been made by THOMSON and KLIPFEL (1957), who have derived for mitochondria an empirical equation, based on the experimental data of TEDESCHI and HARRIS (1955) and on direct measurements of particle density by the isopycnic method.* According to these authors, a more rigorous treatment leads to an entirely unmanageable equation. As will be shown presently a simple equation can be worked out by introducing a few minor approximations.

It is first supposed that the particles behave as perfect osmometers and that no interactions occur between solvent and solute either in the suspension medium or in the internal solution of the particle. These assumptions have received direct experimental support from the experiments of TEDESCHI and HARRIS (1955) on rat-liver mitochondria suspended in sucrose. The solvent is defined as the physical solvent plus the solutes diffusing through the membrane. The solutes are defined as the non-penetrating solutes only.

Let

ϕ, μ and ρ_p be the volume, the mass and the density of the particle,

ϕ_d, μ_d and ρ_d, the volume, the mass and the density of the particle entirely deprived of solvent,†

α, the amount of osmotically active substances inside the particle, in millimoles per unit mass of the particle entirely deprived of solvent,†

ρ_w, the density of the solvent,

ρ_u, the density of the solutes in solution,

V, the average partial molal volume of the solutes, in cubic centimetres per millimole,

m, the total molal concentration of the solutes (in moles per 1000 g of solvent),

c, their total molar concentration (in moles per 1000 cm^3 of solution).

* This equation is of the form of equation (4), with r_0 replaced by a quadratic and ρ_p by a linear function of the osmotic pressure (sucrose concentration). In fact the true relationship between particle density and osmotic pressure has a fairly pronounced curvature and the authors' formula becomes inexact for the higher sucrose concentrations.

† This term is preferred to that of dry particle, to account for the possibility that the framework of the particle may contain a certain amount of bound water (water of hydration), which does not participate in the osmotic movements, at least within the range of osmotic pressure considered.

Osmotic equilibrium is obtained when the mole fraction of solvent is the same inside and outside the particles. The amount of solvent taken up by the particle will then be:

$$\frac{\alpha\mu_d}{\rho_w m}\ \text{cm}^3 \quad \text{or} \quad \frac{\alpha\mu_d}{m}\ \text{g}$$

Thus:

$$\phi = \phi_d\left(1 + \frac{\rho_d\alpha}{\rho_w m}\right) \qquad \ldots.(7)$$

$$\mu = \mu_d\left(1 + \frac{\alpha}{m}\right) \qquad \ldots.(8)$$

$$\rho_p = \rho_d\rho_w\frac{\alpha + m}{\rho_d\alpha + \rho_w m} \qquad \ldots.(9)$$

From the assumption that no interactions occur between the solvent and the solutes, the density of the solution and its molality are related by:

$$\rho_m = \rho_w\frac{1 + \rho_u V m}{1 + \rho_w V m} \qquad \ldots.(10)$$

This formula is very closely followed by sucrose in aqueous solutions with $V = 0{\cdot}21$ and $\rho_u = 1{\cdot}63$.

An equivalent set of formulas could be written using the molarity scale c instead of the molality m, by the transformation (valid in the absence of solvent–solute interactions):

$$m = \frac{c}{\rho_w(1 - Vc)} \qquad \ldots.(11)$$

Combination of equations (7), (9) and (10) with equation (2) leads to an expression of the sedimentation constant s as a function of the density of the medium:

$$s = \frac{\phi_d}{f}[\rho_d(1 - \rho_u\alpha V) - \rho_m(1 - \rho_d\alpha V)] \qquad \ldots.(12)$$

It is also found from equations (9) and (10) that particles and medium have the same density when:

$$\rho_p = \rho_m = \rho_e = \rho_d\frac{1 - \rho_u\alpha V}{1 - \rho_d\alpha V} \qquad \ldots.(13)$$

$$m_e = \frac{\rho_d(1 - \rho_u\alpha V) - \rho_w(1 - \rho_d\alpha V)}{\rho_w(\rho_u - \rho_d)V} \qquad \ldots.(14)$$

in which ρ_e is the equilibrium or isopycnic density and m_e the corresponding molal concentration of solutes.

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These equations are useful in the planning or interpretation of isopycnic sedimentation experiments carried out in media of different composition. It is obvious that density equilibrium is theoretically possible only when definite relationships exist between ρ_d, ρ_u, ρ_w and V. For instance let us suppose that $\rho_u > \rho_d > \rho_w$ as is usually the case. The curves ρ_p and ρ_m v. m will intersect at finite m differing from 0, if

$$V < \frac{\rho_d - \rho_w}{\rho_d \alpha(\rho_u - \rho_w)}$$

This intersection at ρ_e will occur at lower concentrations for larger or denser molecules or solutes. With very big molecules, such that the inequality given above does not hold, an equilibrium density will be obtained only if the solute has a lower specific gravity than the dehydrated granules: $\rho_d > \rho_u > \rho_w$. The particular case, $\rho_u \alpha V = \rho_d \alpha V = 1$, is worth mentioning: the particles and the medium have the same density at all concentrations, as shown by comparing equations (9) and (10).

In differential centrifugation it is convenient to have equation (12) in an abridged form, obtained by introducing equation (13):

$$s = \frac{\phi_d}{f}(1 - \rho_d \alpha V)(\rho_e - \rho_m) \qquad \ldots.(15)$$

or, by analogy with equation (2):

$$s = \frac{\phi'}{f}(\rho_e - \rho_m) \qquad \ldots.(16)$$

where ϕ', the *apparent volume* of the particle is:

$$\phi' = \phi_d(1 - \rho_d \alpha V) \qquad \ldots.(17)$$

and ρ_e, its *apparent density*, is in fact the isopycnic density. It will be noticed that these constants do not depend exclusively on the properties of the particles but vary also with the properties of the solutes; on the other hand they are independent of the density of the solvent.

In view of equations (3) and (5), equation (16) may also be written:

$$s = \frac{\phi'(\rho_e - \rho_m)}{6\pi\eta r_o \theta} \qquad \ldots.(18)$$

It still therefore contains two terms, r_o and θ, which are affected by the osmotic pressure. However, the modifications of these terms are much smaller than those already corrected for and, in addition, often occur in opposite directions. For instance, it is known that mitochondria have a rod-like shape in hypertonic solutions of sucrose and

are spherical in an isotonic medium. Therefore, the decrease of r_o brought about by osmotic dehydration is compensated to some extent by an increase of θ. If, therefore, the product $r_o\theta$ is taken to remain approximately constant, equation (18) may finally be written:

$$s = \frac{\phi'}{k} \cdot \frac{(\rho_e - \rho_m)}{\eta} \qquad \ldots.(19)$$

in which

$$k = 6\pi r_o\theta \qquad \ldots.(20)$$

In view of the suggestion by WERKHEISER and BARTLEY (1957) that mitochondria may contain a "sucrose space" (see, however, AMOORE and BARTLEY, 1958), it seemed of interest to examine also the case of particles possessing a compartment permeable to the external solutes. In order to conserve the same notations, it will be assumed that to the perfect osmotic system defined above is annexed an additional constant volume defined as $\phi_m = \beta\phi_d$ of density ρ_m, thus equal at all times to the density of the medium. Then equations (7), (8) and (9) become:

$$\phi = \phi_d\left(1 + \beta + \frac{\rho_d\alpha}{\rho_w m}\right) \qquad \ldots.(7')$$

$$\mu = \mu_d\left(1 + \frac{\rho_m}{\rho_d}\beta + \frac{\alpha}{m}\right) \qquad \ldots.(8')$$

$$\rho_p = \rho_d\rho_w \frac{\alpha + \left(1 + \frac{\rho_m}{\rho_d}\beta\right) m}{\rho_d\alpha + (1 + \beta)\rho_w m} \qquad \ldots.(9')$$

These equations are more general than the former ones, which can be derived from them by making $\beta = 0$. On the other hand, if α is made $= 0$ in them, one obtains the properties of an entirely permeable particle of constant volume. Of special practical interest is the fact that equations (12) and (13) and all the following derived from them remain valid. In other words, both the sedimentation constant and the equilibrium density are independent of the presence in the particle of a space permeable to the external solutes, provided this space equilibrates sufficiently rapidly with the outside medium. However the existence of such a space will affect the calculation of particle size from centrifugation data by means of equations (4) or (6), since equation (9') will have to be used instead of equation (9).

Applications to rat-liver mitochondria

(*a*) *Physical properties.* In a valuable piece of work, involving actual measurements of mitochondrial volumes, TEDESCHI and HARRIS (1955) have shown that rat-liver mitochondria obey equation (7) or (7')

over a fairly wide range of sucrose concentrations. From the data of their Fig. 3, it may be derived that the average values for the constants are

$$\phi_d(1 + \beta) = 8{\cdot}5 \times 10^{-14} \text{ cm}^3$$
$$\rho_d \alpha \phi_d = 3{\cdot}2 \times 10^{-14} \text{ millimoles}$$

Another experimental value is that of ρ_e, which is reported by THOMSON and MIKUTA (1954) to be equal to 1·22 in pure sucrose solutions. This value has been confirmed in this laboratory; it corresponds to a molal concentration $m_e = 2{\cdot}57$ (with $\rho_u = 1{\cdot}63$ and $V = 0{\cdot}21$ in the range of concentration considered).

From these data, the following average values may be derived by the above equations (assuming $\beta = 0$):

$$\alpha = 0{\cdot}300 \text{ millimole g}^{-1}$$
$$\phi_d = 8{\cdot}5 \times 10^{-14} \text{ cm}^3$$
$$\mu_d = 10{\cdot}65 \times 10^{-14} \text{ g}$$
$$\rho_d = 1{\cdot}252 \text{ g cm}^{-3}$$

and for mitochondria in 0·25 M sucrose:

$$\phi = 20{\cdot}6 \times 10^{-14} \text{ cm}^3$$
$$\mu = 22{\cdot}75 \times 10^{-14} \text{ g}$$
$$\rho_p = 1{\cdot}103 \text{ g cm}^{-3}$$
$$r_o = 3{\cdot}66 \times 10^{-5} \text{ cm}$$

THOMSON and co-workers (1954, 1956) and KUFF *et al.* (1956) estimated the radius of mitochondria from measurements of s. However, these authors used ρ_e to represent ρ_p in equation (6) and assumed ϕ to be invariable. Consequently the particle radii they reported correspond in fact to $r' = \sqrt{(9\phi'\theta/2k)}$. From the above data $r' = 2{\cdot}3 \times 10^{-5}$ cm is obtained, and this corresponds closely to the experimental values reported. Later results of THOMSON and KLIPFEL (1957) were corrected for the osmotic changes in volumes, and gave $r_o = 3{\cdot}75 \times 10^{-5}$ cm for the mitochondria. The agreement between the direct measurement of the mitochondrial radius and its estimation from sedimentation experiments indicates that the theoretical treatment given above represents a good approximation to the real situation.*

(*b*) *Isopycnic media.* The data computed above make it possible to calculate the properties of the solutions in which rat-liver mitochondria

* In a recent paper, THOMSON and KLIPFEL (1958) have reported values of $r_o = 3{\cdot}5 \times 10^{-5}$ cm and $\rho_p = 1{\cdot}14$ g cm^{-3} for mitochondria suspended in 0·25 M sucrose containing variable amounts of polyvinylpyrrolidone. The authors claim that the polymer has negligible osmotic effects, but this assumption is open to question in view of the fact that mitochondria maintain their rod-like shape in 0·25 M sucrose containing 7·3 per cent polyvinylpyrrolidone (NOVIKOFF, 1957).

will have the same density as in 0·25 M sucrose (0·265 molal), while being isopycnic with the surrounding medium. This is done most easily by making $\rho_e = 1{\cdot}103$ in equation (13), which gives V as a function of ρ_u. In Table 2 are listed a number of representative values for aqueous solutions. Attention is called to the values given for $\rho_u = 1{\cdot}25$, which characterize the solute whose solutions are isopycnic with mitochondria at all concentrations compatible with the structural integrity of these bodies.

TABLE 2

Properties of 0·265 Molal Solutions Isopycnic with Rat-liver Mitochondria

Properties of solute			*Concentration of solution*	
Density ρ_u	Molal volume V	Mol. wt.	Mole/l. c	% (w/w)
1·15	8·44	9710	0·082	72·0
1·20	4·09	4910	0·127	56·5
1·25	2·70	3380	0·154	47·2
1·35	1·61	2170	0·186	36·5
1·63	0·75	1220	0·221	24·5
2·00	0·44	880	0·237	18·9
3·00	0·209	630	0·251	14·3
4·00	0·137	550	0·256	12·7
5·00	0·102	510	0·258	11·9
10·00	0·045	450	0·262	10·7

(*c*) *Hydration.* As shown by equation (13), ρ_e is theoretically independent of ρ_w, the density of the solvent. Experiments performed in this laboratory with solutions of sucrose in deuterium oxide have not entirely confirmed this prediction. For mitochondria, the equilibrium density was only slightly higher in heavy water, but the difference was much greater for lysosomes and especially for the uricase-containing particles. These results suggest that the mass μ_d includes a certain amount of water of hydration, which can exchange with the heavy water of the medium. It is for this reason that the particle constants were referred to the particle entirely deprived of solvent rather than to the dry particle (see footnote, p. 333).

3.4. *Influence of temperature.* The most important temperature-sensitive factor in the rate equation is the viscosity of the medium. For instance, between 0° and 5°, the viscosity of sucrose solutions decreases by a factor which varies from 15 per cent to more than 30 per cent, depending on the concentration (see below, Table 3). A close check on the temperature is therefore essential in all measurements

of sedimentation rate. This factor is of less importance in pure density equilibration.

3.5. *Influence of particle concentration.* At present, no information is available concerning the influence of concentration on the sedimentation of cell particles. In the sedimentation of proteins and other macro-molecules, this influence is known to be quite important and it seems likely that the same will be true for cell particles. Preliminary experiments in this laboratory have indeed shown that the sedimentation of rat-liver mitochondria is concentration-dependent. However, the exact form of the relationship has not yet been worked out.

3.6. *Reduction of sedimentation constant to standard conditions.* It is customary in analytical centrifugation to reduce the observed sedimentation constants to standard conditions (pure water; 20°; infinite dilution). The same appears to be desirable in the case of cell particles, but more suitable standard conditions should be chosen. It is accordingly proposed to define the standard sedimentation constant as the value of s in isotonic sucrose at 0°C and to refer to this value as s_s. In order to avoid confusion and since the sucrose solutions used are generally prepared at room temperature, isotonic sucrose will be defined as a 0·25 M solution at 20°, i.e. 0·265 molal or 8·29 per cent by weight (for properties of sucrose solutions, see Table 3).

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According to equation (19), reduction of s to s_s may be effected by the following transformation:

$$s_s = s \frac{\phi'_s(\rho_{es} - \rho_{ms})k\eta}{\phi'(\rho_e - \rho_m)k_s\eta_s} \qquad \ldots.(21)$$

in which the subscript s refers in all cases to the values in 0·25 M sucrose at 0°, except for ρ_{es} which is the equilibrium density in sucrose. Introducing this transformation in the velocity equation (2), we obtain finally the general formula which will be used in all the discussions of the second part:

$$v = Bs_s\omega^2 \frac{\rho_e - \rho_m}{\eta} x \qquad \ldots.(22)$$

in which

$$B = \frac{\phi' k_s \eta_s}{\phi'_s k(\rho_{es} - \rho_{ms})} \qquad \ldots.(23)$$

If the experiments are performed in sucrose, this expression reduces to:

$$B = \frac{k_s\eta_s}{k(\rho_{es} - \rho_{ms})} \qquad \ldots.(24)$$

or

$$B = \frac{\eta_s}{\rho_{es} - \rho_{ms}} = \frac{0{\cdot}0228}{\rho_e - 1{\cdot}0341} \qquad \ldots.(25)$$

if, as will usually be the case, the ratio of the two ks has to be assumed equal to unity.

If another solute is used, equation (23) has to be applied, together with equations (13) and (17), for ϕ' and ρ_e are functions of the partial molal volume of the solute. The computation of B in this case requires knowledge of ρ_d and α.

It should be noticed that the above equations do not take into account the influence of the particle concentration, as it is not known at the present time. Eventually, it may become necessary to redefine the standard sedimentation constant as the value of s_s at infinite dilution.

4. *Diffusion*

As a rough approximation and in dilute solution, the diffusion constant of a spherical particle is inversely proportional to the square root of its sedimentation constant. Thus, the diffusion constant of mitochondria may be estimated to be about 100 times smaller than that of a protein of mol. wt. 100,000. Although very small, this factor may not be entirely negligible under some conditions, such as isopycnic sedimentation where strong gradients of particle concentration are built up; it becomes proportionately more important in investigations on smaller particles and on microsomes. For practical reasons, it will be neglected in the following discussions, since introduction of a diffusion factor into the equations for density gradient centrifugation would complicate the problem to a considerable extent. In practice, the populations analysed will appear as somewhat more polydisperse than they actually are.

5. *Convection*

The main causes of convection are changes in temperature, local changes in density caused by side-wall impaction, abnormal sedimentation in layered systems, as studied by ANDERSON (1955a) and swirling during acceleration and deceleration of the centrifuge. A theory for sedimentation with complete convective mixing has been developed by BECHHOLD and SCHLESINGER (1931). Present improvements in equipment make it unnecessary to take this factor into account in the calculations, provided adequate precautions are taken to minimize convective disturbances. These will be examined in the third section of this paper. It should be mentioned also that the existence of a density gradient in the centrifuge tube greatly stabilizes against such disturbances.

IV. Analysis of Distribution Curves

1. *General equations*

The preceding equations describing the rate of sedimentation of particles in a centrifuge can be applied to the analysis of their distribution after

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centrifugation. The centrifuge tube is supposed to have its axis perpendicular to the axis of rotation. Let us consider an element of surface of area a located at a radial distance x and normal to the radius; another element of area $a + da$ located on the same radius at $x + dx$. The two elements are related to each other in such a way that all the particles crossing the first will cross the other a little later. If the particles follow parallel directions (parallel sedimentation), $da = 0$. If the particles follow exactly the direction of the centrifugal force (radial sedimentation), $da = a\,dx/x$.

In a cylindrical centrifuge tube, the equivalent of pure radial sedimentation occurs only if all the particles which hit the sidewalls slide down to the bottom very rapidly. As shown above (see p. 330), this may not be the true situation and the divergence of the freely moving particles may be compensated to an unknown extent by a rebound of particles from the sidewalls. Let γ be the fraction of particles which after side-wall impaction sediment immediately, $1 - \gamma$ the fraction bouncing back into the fluid, then da, as defined above, is equal, *on an average* and for a given level x, to $a\gamma\,dx/x$. In the following deductions, γ will be treated as a constant, which is probably not rigorously correct.

Let C be the particle concentration and v, the velocity of sedimentation, at radial distance x and time t. The number of particles crossing the section area a during an interval dt is $Cva\,dt$.

Similarly the total number of particles crossing the area $a + da$ at level $x + dx$ is:

$$\left(C + \frac{\delta C}{\delta x}\,dx\right)\left(v + \frac{\delta v}{\delta x}\,dx\right)\left(1 + \gamma\,\frac{dx}{x}\right)a\,dt$$

The difference between these two expressions represents the increase over interval dt of the number of particles present in volume $a\,dx$, so that:

$$-\left[v\,\frac{\delta C}{\delta x} + C\,\frac{\delta v}{\delta x} + \frac{Cv\gamma}{x}\right]a\,dx\,dt = a\,\frac{\delta C}{\delta t}\,dx\,dt$$

The differential equation is therefore:

$$\frac{\delta C}{\delta t} + v\,\frac{\delta C}{\delta x} = -\,C\left(\gamma\,\frac{v}{x} + \frac{\delta v}{\delta x}\right) \qquad \ldots.(26)$$

The solution $C(x,t)$ obviously satisfies the relation:

$$\frac{\delta C}{\delta t}\,dt + \frac{\delta C}{\delta x}\,dx = dC$$

which gives by comparison with equation (26):

$$dt = \frac{dx}{v} = -\frac{dC}{C\left(\gamma\frac{v}{x} + \frac{\delta v}{\delta x}\right)} \qquad \ldots.(27)$$

It is theoretically possible to calculate the function $C(x,t)$ from equation (27) and the initial conditions of the problem, but only in the simplest cases does one arrive at an algebraically manageable solution. In practice, knowledge of this function is not essential and all the needed information can be obtained by integrating between limits the two equations (27), in which v will have been replaced by its value.

If, as is inevitable at the present time (see p. 339), the influence of particle concentration on the velocity of sedimentation is neglected, v is a function of x only and may be written in all cases (see equations 2 and 22):

$$v = s_s\omega^2 u(x) \qquad \ldots.(28)$$

Then, by integration of equation (27), we obtain:

$$\int_{x_i}^{x}\frac{dx}{u(x)} = s_s\int_0^t \omega^2\,dt = s_s W \qquad \ldots.(29)$$

$$\frac{C}{C_i} = \left(\frac{x_i}{x}\right)^{\gamma}\frac{u(x_i)}{u(x)} \qquad \ldots.(30)$$

The physical meaning of these equations is the following: let x_i and C_i be the level and the concentration of particles at time $t = 0$; x and C will be their position and concentration at time t. Thus, x is no longer a truly independent variable, but it represents the level reached at time t by a given group of particles occupying an initial position x_i. Equation (29) can be called accordingly the *position equation*; in particular, when x_i is the radial distance of the meniscus x_o, equation (29) gives the position of the boundary as a function of time. Equation (30) gives the concentration C of a group of particles at the level x, after they have travelled from the level x_i, where they were at concentration C_i; it will be called the *concentration equation*.

2. *Applications*

2.1. *Monodisperse suspension in a homogeneous medium.* This case has been treated exhaustively before (see for instance: SVEDBERG and PEDERSEN, 1940; NICHOLS and BAILEY, 1949), and will be recalled here only as an introduction to the more complex problems of sedimentation in concentration gradients.

Comparison of equations (2) and (28) indicates that $s_s u(x) = sx$. Thus, equations (29) and (30) become:

$$sW = \ln \frac{x}{x_i} \qquad \ldots.(31)$$

$$\frac{C}{C_i} = \left(\frac{x_i}{x}\right)^{1+\gamma} \qquad \ldots.(32)$$

In this simple case x_i can be eliminated easily between equations (31) and (32) to give C as function of time below the boundary:

$$\ln \frac{C}{C_i} = -(1 + \gamma)sW \qquad \ldots.(33)$$

In Fig. 1 is shown the behaviour of a monodisperse suspension homogeneously distributed in a horizontal tube, while Fig. 2 illustrates

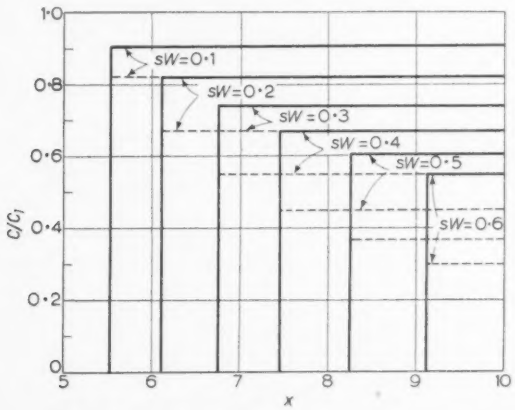

Fig. 1. Theoretical sedimentation diagram of a monodisperse suspension in a homogeneous medium. Radial distance of meniscus = 5 cm; diffusion effects omitted. Full lines, $\gamma = 0$; broken lines, $\gamma = 1$.

the fate of a similar layered system. The boundary values are estimated by means of equation (31), the concentrations by means of equation (32) with the limit values of γ (0 and 1).

The proportion of completely sedimented particles can also be predicted by means of the above equations. In the case of a homogeneously filled, flat-bottomed,* cylindrical tube, the percentage of completely sedimented particles is given by:

$$\text{per cent sed.} = \frac{C_i(x_f - x_o) - C(x_f - x_b)}{C_i(x_f - x_o)} \cdot 100 \qquad \ldots.(34)$$

* It is convenient in such calculations to consider spherical-bottomed tubes as equivalent to flat-bottomed tubes of equal volume, thus shorter by one-third the radius.

in which

x_f = the radial distance of the bottom of the tube,

x_o = the radial distance of the meniscus,

x_b = the radial distance of the boundary.

Replacing C and x_b by their values, one obtains:

$$\text{per cent Sed} = \left[1 - \frac{x_f \exp\left[-(1+\gamma)\, sW\right] - x_o \exp\left(-\gamma sW\right)}{x_f - x_o}\right] . 100 \quad \ldots . (35)$$

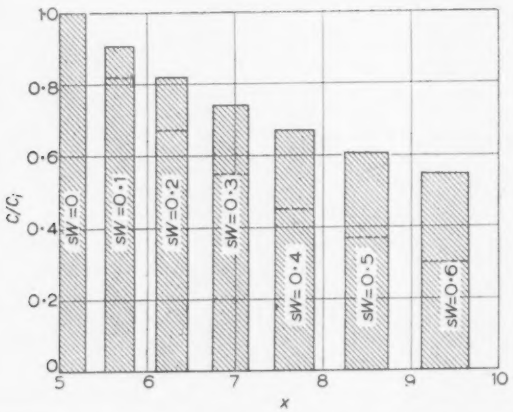

Fig. 2. Theoretical sedimentation diagram of a monodisperse suspension layered over a homogeneous medium. Thickness of layer = 0·3 cm; radial distance of meniscus = 5 cm; diffusion effects omitted. Full lines, $\gamma = 0$; broken lines, $\gamma = 1$.

Complete sedimentation occurs when $x_o \exp sW = x_f$, i.e. when the boundary reaches the bottom of the tube. In Fig. 3 the percentage of sedimented particles has been plotted as a function of the product sW for values of $x_o = 5$ and $x_f = 10$ cm. Such charts, adapted to the type of centrifuge used, illustrate the rate of accretion of the sediment as a function of time, when a monodisperse suspension is centrifuged at constant speed (sW = constant × time). They also may serve to estimate the contamination of a sediment in conventional differential centrifugation on polydisperse suspensions. If, for instance, centrifugation has been performed at a value of W equal to 7×10^8 rad^2 sec^{-1}, then all particles of sedimentation constant equal to or higher than $10^4 S$ will be completely sedimented. As pointed out before (DE DUVE and BERTHET, 1954), the contamination of the sediment is 40 per cent and 13 per cent for particles with sedimentation constant one-third and one-tenth the above value respectively (assuming $\gamma = 0$). If γ is taken to be equal to one, the contamination actually reaches 53 and

20 per cent. It will also be noticed that if $\gamma > 0$, a sediment will form in a layered system before the lower layer boundary has reached the bottom of the tube.

2.2. *Polydisperse suspension in a homogeneous medium.* Let C_i be the total concentration of particles in the original suspension; C_{si}, the concentration of those having a sedimentation constant smaller or equal to s. The fraction of particles having a sedimentation constant

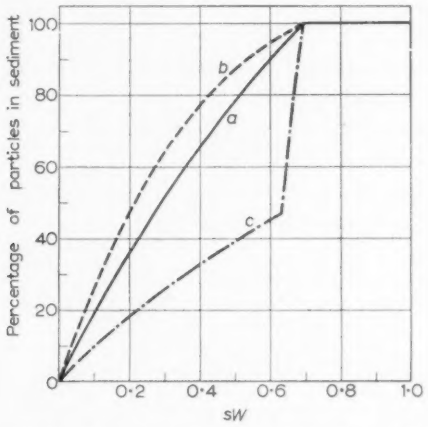

Fig. 3. Sedimentation of a monodisperse suspension. Tube dimensions: $x_o = 5$ cm, $x_f = 10$ cm. Completely filled tube: curve a, $\gamma = 0$; curve b, $\gamma = 1$; curve c: layered system 0·3 cm thick, $\gamma = 1$.

between s and $s + \mathrm{d}s$ is $\mathrm{d}C_{si}/C_i$. The frequency curve of the sedimentation constants is defined as

$$F(s) = \frac{1}{C_i} \cdot \frac{\mathrm{d}C_{si}}{\mathrm{d}s} \qquad \ldots\ldots(36)$$

The problem in analysing a sedimentation diagram of cell particles is to find the function $F(s)$ from the distribution of the particles $C(x)$ in the tube after centrifugation.

After centrifuging a homogeneously filled tube for W rad^2 sec^{-1}, the particles starting from the meniscus x_o and with sedimentation constant between s and $s + \mathrm{d}s$ will be located between $x = x_o \exp(sW)$ and $x + \mathrm{d}x = x + Wx\,\mathrm{d}s$ (according to equation 31). In the zone $\mathrm{d}x$ the particles in the range $\mathrm{d}s$ will have a concentration $\mathrm{d}C_s = \mathrm{d}C_{si}(x_o/x)^{1+\gamma}$ (according to equation 32). All the particles with sedimentation constants lower than s or higher than $s + \mathrm{d}s$ are at uniform or zero concentration over the zone $\mathrm{d}x$; thus $\mathrm{d}C_s = \mathrm{d}C$; division by $\mathrm{d}x = Wx\,\mathrm{d}s$ yields:

$$\frac{\mathrm{d}C}{\mathrm{d}x} = \frac{1}{Wx}\left(\frac{x_o}{x}\right)^{1+\gamma}\frac{\mathrm{d}C_{si}}{\mathrm{d}s} \qquad \ldots\ldots(37)$$

The frequency function can thus be computed from a sedimentation pattern $C(x)$ by:

$$F(s) = Wx\left(\frac{x}{x_o}\right)^{1+\gamma}\frac{1}{C_i}\frac{dC}{dx} \qquad \ldots.(38)$$

Attention is called to the significance of the various terms in equation (38), which may be rewritten as the product of four factors:

$$F(s) = \frac{1}{C_i}\frac{dC}{dx}\cdot\frac{x}{x_o}\cdot\left(\frac{x}{x_o}\right)^{\gamma}\cdot Wx \qquad \ldots.(38')$$

It can then be seen that the function $1/C_i \,.\, dC/dx$ gives $F(s)$ after correction by the following factors:

x/x_o which corrects for the radial spreading due to the increase of centrifugal field with distance;

$(x/x_o)^{\gamma}$ which corrects for the lateral spreading (geometrical correction)

Wx which ensures conversion from the x to the s scale.

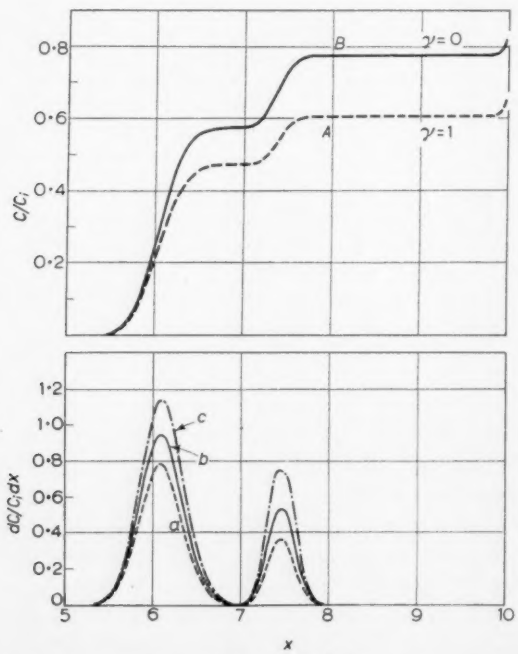

Fig. 4. Analysis of sedimentation diagram of polydisperse suspension of Fig. 5. Radial distance of meniscus = 5 cm; $W = 10^8$ rad^2 sec^{-1}. Upper part, sedimentation diagram: curve A, $\gamma = 1$; curve B, $\gamma = 0$. Lower part, derivatives: curve a, derivative of A; curve b, derivative of B or a corrected for geometrical dilution; curve c, b corrected for radial spreading. With $C/C_i\ dl$ as ordinate, b shows sedimentation diagram of infinitely thin layer with $\gamma = 1$; c shows same curve corrected for geometrical loss or corresponding diagram for $\gamma = 0$.

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These various steps in the computation of distribution curves are illustrated in Fig. 4. Curves A and B represent the sedimentation diagrams which would be obtained by centrifuging at a value of $W = 10^8$ rad^2 sec^{-1} the hypothetical bimodal population represented in Fig. 5. A is the curve for $\gamma = 1$, B for $\gamma = 0$. Underneath are shown the corresponding derivatives. Curve a is the derivative of curve A. After geometrical correction, it becomes b, which is also the derivative of curve B. After further correction for radial spreading, curve c is

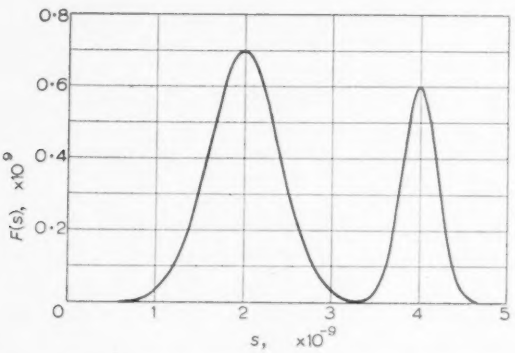

Fig. 5. Frequency curve of hypothetical bimodal population showing diagrams of Fig. 4.

obtained, which after suitable conversion to the s-scale becomes the distribution curve of Fig. 5.

In practice, the analysis of sedimentation diagrams proceeds stepwise. Finite increments $\Delta C/C_i$ are measured over successive intervals Δx, often corresponding to the actual sampling cuts taken in the column of fluid. Mean values of x and s are used to calculate the abscissa and ordinate values of the distribution curve by means of equations (31) and (38).

If the suspension at concentration C_i has been deposited as an infinitely thin layer $\mathrm{d}l$ on the top of the tube, the curve $C(x)$ itself gives the function $F(s)$ after suitable correction. The amount of particles in the original suspension with sedimentation constant in the range s to $s + \mathrm{d}s$ is $C_i\,\mathrm{d}l\,F(s)\,\mathrm{d}s$. After centrifugation, the fraction $(x_o/x)^\gamma$ of these particles which have not been lost by side-wall impaction is spread over a distance $\mathrm{d}x$, such that $W\,\mathrm{d}s = \mathrm{d}x/x$. Thus:

$$C(x)\,\mathrm{d}x = \left(\frac{x_o}{x}\right)^\gamma C_i\,\mathrm{d}l\,F(s)\,\mathrm{d}s = \left(\frac{x_o}{x}\right)^\gamma \frac{C_i\,\mathrm{d}l}{Wx}\,F(s)\,\mathrm{d}x$$

and

$$F(s) = Wx\left(\frac{x}{x_o}\right)^\gamma \frac{C(x)}{C_i\,\mathrm{d}l} \qquad \ldots.(39)$$

With the F functions of Fig. 5, the sedimentation diagrams would be b or c of Fig. 4, with $\gamma = 1$ or 0 respectively. The ordinate of Fig. 4 should then be $C/C_i\ \mathrm{d}l$.

In actual experiments, the layer of suspension deposited is fairly thick and equation (39) gives only an approximate result.

2.3. *Monodisperse suspension in a concentration gradient of solutes.* The formal solution of the problem is given by equations (29) and (30), in which an appropriate function $u(x)$ has been introduced. From equation (22), it is seen that:

$$u(x) = B\,\frac{\rho_e - \rho_m}{\eta} \cdot x \qquad \ldots.(40)$$

in which B and ρ_e are properties of the particles studied and of the solutes used (see equation 13), while ρ_m and η are now functions of x.

None of the examples in the literature has yet been treated exactly in the manner described here, but integrals very similar to equation (29) have been computed by THOMSON and MIKUTA (1954) and by KUFF *et al.* (1956). Linear equations were used for ρ_m by both groups and for η by KUFF *et al.*, while a cubic function of x was necessary to represent the viscosity in the experiments of the Thomson group, who used a steep gradient.

Actually, the most convenient form of $u(x)$ is of the type:

$$u(x) = \frac{B}{n + px + qx^2 + rx^3 \ldots} \qquad \ldots.(41)$$

which is easily obtained by fitting a polynomial $n + px + qx^2 + rx^3$ to the function $\eta/[(\rho_e - \rho_m)x]$. In practice a sufficient approximation will be provided in all cases by a cubic function, sometimes even by a quadratic or linear function when the gradient is not too steep.

The next step in the calculations is to compute

$$\Phi(x) = \int \frac{\mathrm{d}x}{u(x)} = \frac{1}{B}\left(nx + \frac{p}{2}x^2 + \frac{q}{3}x^3 + \frac{r}{4}x^4\right) \qquad \ldots.(42)$$

and

$$\Psi(x) = x^{\gamma}u(x) \qquad \ldots.(43)$$

and to plot them as a function of x. These two graphs are called the reference graphs of the gradient for the particles of given B and ρ_e.

The position and concentration equations take the form

$$s_g W = \Phi(x) - \Phi(x_i) \qquad \ldots.(44)$$

and

$$C = C_i\,\frac{\Psi(x_i)}{\Psi(x)} \qquad \ldots.(45)$$

It is not possible or not convenient to eliminate x_i between these two equations to obtain $C(x,s_gW)$; the graphs Φ and Ψ are used in the com-

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putation of a sedimentation diagram. From the graph Φ, the level x reached by particles is obtained for given values of x_i and $s_s W$; the value of x is used to compute C with the aid of graph Ψ. By plotting C v. x the sedimentation diagram is obtained. In Figs. 6 and 8 are

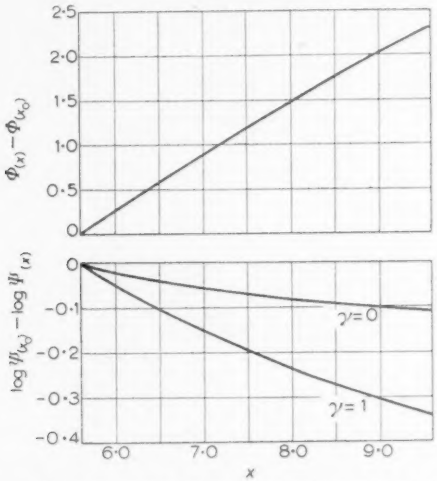

Fig. 6. Reference graphs of gradient of KUFF *et al.* (1956).

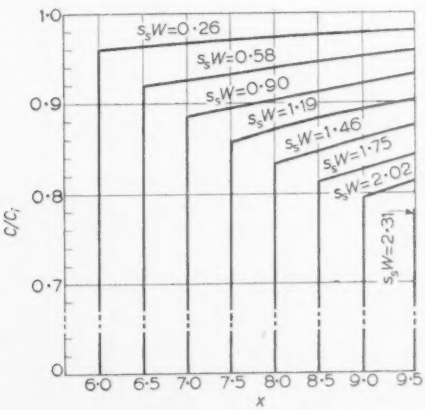

Fig. 7. Sedimentation diagrams of monodisperse suspension in gradient of KUFF *et al.* (1956).

shown the reference graphs for rat-liver mitochondria of the gradients used by KUFF *et al.* (1956) and by THOMSON and co-workers (1954, 1956, 1957), as computed from the values given by these authors. A few theoretical sedimentation diagrams in these sucrose gradients are represented in Figs. 7 and 9. It is seen that the concentrations do not remain constant below the boundary, as was the case in Fig. 1.

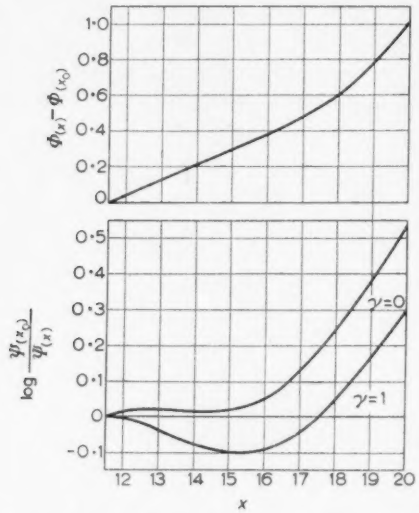

Fig. 8. Reference graphs of gradient of THOMSON and co-workers (1954, 1956, 1957).

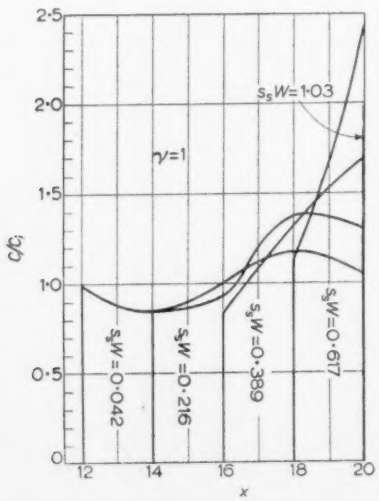

Fig. 9. Sedimentation diagrams of monodisperse suspension in gradient of THOMSON and co-workers (1954, 1956, 1957).

This method of calculation was applied to predict the theoretical behaviour of mitochondria during an isopycnic sedimentation experiment. In Fig. 10 are shown the reference graphs of a sucrose gradient used in this laboratory for the analysis of mitochondrial fractions. The sucrose concentration varied linearly from a value of from 1·43 M [41·1

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per cent (w/w)] to 1·895 M [51·9 per cent (w/w)] over a radial distance of 5·5–9·6 cm. The value of ρ_e of mitochondria as determined in these experiments, was 1·216. The particles were evenly distributed in the whole tube at the beginning of the centrifugation.

Fig. 11 illustrates the theoretical sedimentation diagrams for various values of s_sW. These values actually correspond to minutes of

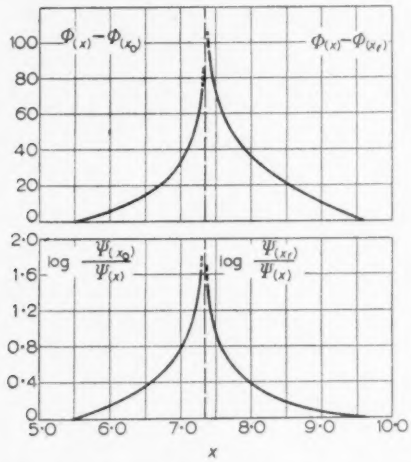

Fig. 10. Reference graphs of gradient for isopycnic experiments. Linear concentration gradient of 41·1–51·9 per cent (w/w) aqueous sucrose over 5·5–9·6 cm; $\gamma = 0$.

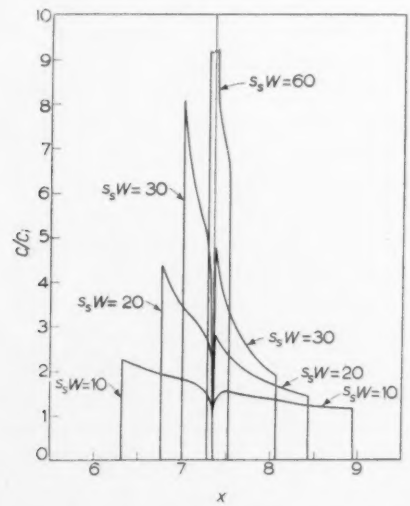

Fig. 11. Sedimentation diagrams of monodisperse suspension in gradient of Fig. 10.

centrifugation at 39,000 rev/min for particles of standard sedimentation constant equal to $10^4\,S$ ($r_o = 0{\cdot}39\ \mu$ if $\rho_p = 1{\cdot}103$ in 0·25 M sucrose). The diagram therefore shows that such systems must be centrifuged for at least 1 hr at 39,000 rev/min for mitochondria to approach density equilibrium. After such a time, particles of one-third the sedimentation constant ($r_o = 0{\cdot}22\ \mu$ for the same density) will still be very far from equilibrium and in addition will show a concentration maximum at a density distinctly lower than the equilibrium density.

2.4. *Polydisperse suspension in a concentration gradient of solutes.* By a reasoning similar to that applied to the analysis of a sedimentation diagram in a homogeneous medium (see p. 345) but based on the general equations (29) and (30), we find:

$$F(s_s) = W \cdot \left(\frac{x}{x_o}\right)^{\gamma} \cdot \frac{[u(x)]^2}{u(x_o)} \cdot \frac{1}{C_i} \frac{\mathrm{d}C_s}{dx} \qquad \ldots.(46)$$

This equation is used to compute the ordinates $F(s_s)$ of the frequency curve, while the corresponding abscissa values are calculated by means of equation (44). However, the fact that the concentration of particles does not remain constant behind the boundary (see Figs. 7 and 9) introduces an additional complication, because $\mathrm{d}C_s$ can no longer be considered equal to $\mathrm{d}C$. A rigorous analysis of the sedimentation diagram must therefore proceed stepwise, starting from the meniscus: from the first increment, the sedimentation constant s_s and the concentration C_{si} of the slowest group of particles is computed. Their contributions to the sedimentation diagram for various values of x are calculated and subtracted from the diagram. The procedure is then repeated on the corrected diagram.

In three cases, a simplified treatment of the data is possible.

(*a*) *Slight gradient of concentration.* When the gradient of solutes is not very steep, the theoretical sedimentation diagrams give a concentration of particles which is very nearly constant below the boundary (see Fig. 7).

In first approximation, $\mathrm{d}C_s$ may then be equated with $\mathrm{d}C$ and the frequency curve computed by applying equation (46) directly to the increments of the sedimentation diagram. This principle has been followed by KUFF *et al.* (1956), who, however, have used equation (38) to correct the increments. Thus, their ordinate values suffer by an error in excess which increases as x becomes larger. It should be noted in addition that these authors expressed their results in terms of particle size distribution and did not correct for the osmotic effects (see p. 337).

(*b*) *Layered suspension of particles.* The analysis is also simplified if the experiment is performed with a layer of particle suspension

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surmounting the sucrose gradient. If the layer is supposed to be infinitely thin (of thickness dl),

$$F(s_s) = W \left(\frac{x}{x_o}\right)^{\gamma} u(x) \frac{C(x)}{C_i \, \mathrm{d}l} \qquad \ldots.(47)$$

is theoretically valid (compare with equation 39, p. 347).

In practice $C(x)$ is not known as a continuous function but only by its average values $\bar{C}$ over ranges $\Delta x = x_2 - x_1$ corresponding to $\Delta s_s = s_2 - s_1$. Therefore, equation (47) is applied in an approximate form by replacing:

$$C(x)\,\mathrm{d}x \text{ by } \bar{C}\Delta x$$

$$\left(\frac{x}{x_o}\right)^{\gamma} \text{ by } \left(\frac{x_1 + x_2}{2x_o}\right)^{\gamma}$$

$$\mathrm{d}s_s = \frac{\mathrm{d}x}{W u(x)} \text{ by } \Delta s_s = s_2 - s_1 = \frac{1}{W}[\Phi(x_2) - \Phi(x_1)]\Delta x$$

giving:

$$F(s_s) = \left(\frac{x_1 + x_2}{2x_o}\right)^{\gamma} \frac{\bar{C}\Delta x}{C_i \, \mathrm{d}l \, \Delta s_s} \qquad \ldots.(48)$$

or

$$F(s_s) = \frac{W}{\Phi(x_2) - \Phi(x_1)} \left(\frac{x_1 + x_2}{2x_o}\right)^{\gamma} \frac{\bar{C}}{C_i \, \mathrm{d}l} \qquad \ldots.(48')$$

Equation (48) is that used by THOMSON and co-workers (1954, 1956, 1957). As the authors are well aware, the distributions calculated in this manner were considerably distorted owing to the actual thickness of the layer. In their calculations, they applied an empirical geometrical correction, corresponding to a fractional value of γ (p. 330) and, in their more recent work (THOMSON and KLIPFEL, 1957) they have taken into account the osmotic effects of the medium (p. 337).

(*c*) *A gradient ensuring almost constant sedimentation velocity.* A sucrose gradient, linear (on the molarity scale) and extending from 0·25 to 0·5 M over a radial distance from 5·5 to 9·6 cm, as may be set up in the SW 39 rotor of the Spinco ultracentrifuge, is of particular interest, since it corrects almost perfectly the increase in sedimentation velocity with radial distance for particles similar to mitochondria ($\rho_e = 1{\cdot}22$).

At 0°C the velocity v varies between 44·7 and 47·8 $\times$ $Bs_s\omega^2$, with a mean value of 47 $\times$ $Bs_s\omega^2$. In first approximation:

$$s_s W = 0{\cdot}174(x - 5{\cdot}5)$$

and

$$C = C_i \left(\frac{x_i}{x}\right)^{\gamma}$$

The analysis of the sedimentation diagram of a tube homogeneously filled with particles at $t = 0$, can be carried out with the simple formula

$$F(s_s) = \frac{W}{0{\cdot}174}\left(\frac{x}{5{\cdot}5}\right)^{\gamma}\frac{\mathrm{d}C}{C_i\,\mathrm{d}x}$$

V. Practical Procedures

1. *Production of gradients*

In early work, gradients were produced by superimposing layers of decreasing density and allowing diffusion to smooth down the discontinuities existing at the interfaces. Most authors now prefer continuous gradients, produced by some mechanical device. In general, this consists of a pair of differentially driven syringes containing two stock solutions of different density and connected by some mixing device with a narrow outlet extending to the bottom of the centrifuge tube. The output of the two syringes is so regulated as to cause the proportion of the denser solution in the mixture to increase continuously.

Anderson (1955b) has described a machine, in which the pistons are pushed by rotating driving arms of variable length. This system produces a variety of S-shaped gradients. An elegant instrument has been constructed by Bock, according to a principle developed independently by Bock and Ling (1954) and by Lakshmanan and Lieberman (1954). In this instrument which is shown in Fig. 12, the right-hand syringe injects the denser solution directly into the magnetically stirred light solution contained in the left-hand syringe, which in turn drives the resulting mixture into the centrifuge tube. This device produces a linear gradient when the individual outputs of the two syringes are equal; if these are unequal, the gradient becomes progressively steeper (concave gradient) or flatter (convex gradient), depending on whether the mixing syringe has the greater or the smaller output. If this syringe is replaced by a mixing vessel of constant volume, the gradient becomes exponential.

Kuff *et al.* (1956) use an instrument in which the two syringes are emptied into a common mixing chamber at respectively increasing and decreasing speeds by means of helicoidal cams of variable pitch acting on the pistons. This instrument is shown in Fig. 13.

A machine operating on a similar principle has been constructed in this laboratory (Fig. 14). The cams are designed so as to cause the delivery of the denser solution to increase with constant acceleration from zero to a maximal output D, that of the lighter solution to decrease at the same rate from D to zero, the combined output being constant and equal to D; the cams are mounted on a single axis driven by a motor and move the syringe pistons by means of guided rods kept in

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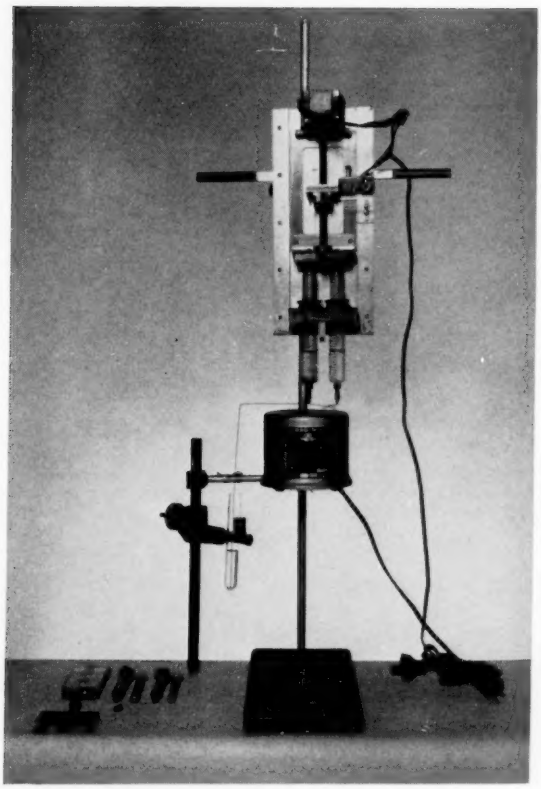

Fig. 12. Gradient making device (by courtesy of Dr. R. M. Bock, Biochemistry Department, University of Wisconsin, Madison).

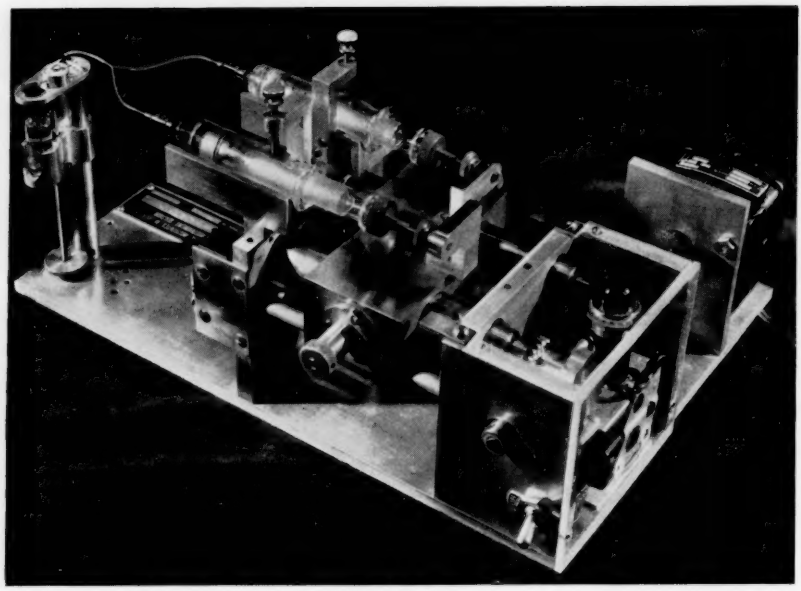

Fig. 13. *Gradient making device* (courtesy of Dr. E. L. KUFF, National Institutes of Health, Bethesda). The apparatus is stopped about half-way in the filling cycle. The more distant syringe, containing the less dense fluid, is further advanced. The two syringes feed into a single polyethylene tubing filled with platinum chips, which create enough turbulence to effect complete mixing.

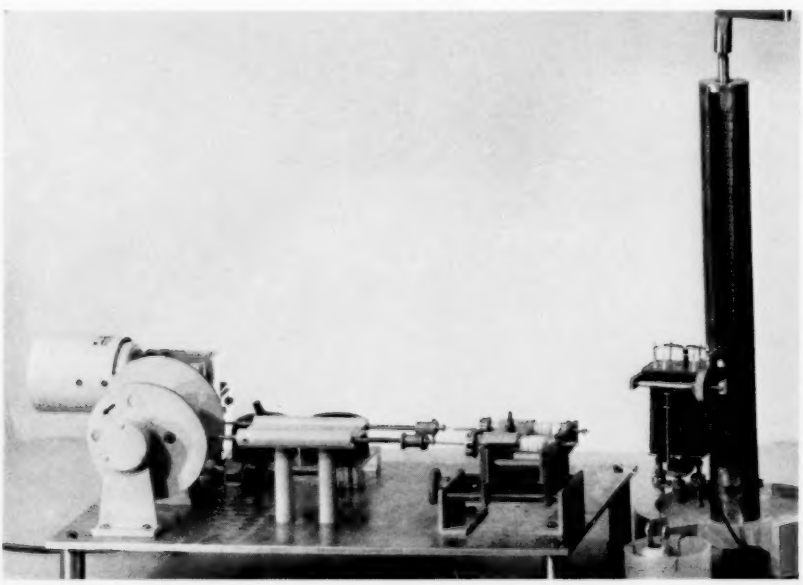

Fig. 14. Gradient making device (authors' model).

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place by coiled springs. The mixing chamber, of very small volume, is closed by an elastic membrane equipped with a miniature stirring rod and connected with an electromagnetic vibrator. When used with identical syringes, the instrument produces a linear gradient. A variety of convex or concave gradients may be obtained with syringes of different cross-sectional areas.

ALBRIGHT and ANDERSON (1958) have constructed a special rotor which permits filling the tubes with the material to be analysed and with the gradient while the centrifuge is running. This disposes of the problems of acceleration (see below), though not of deceleration, and allows very rapid filling (six 200 ml bottles in less than 10 min), since the centrifugal force stabilizes the gradient against mixing. This device is only available so far for low-speed centrifuging. For the preparation of the gradients, the authors use a machine which lowers two Lucite blocks of suitable shape into two vessels containing liquids of different density. The liquid displaced by the lucite blocks is caught in two funnels and conducted to a high-speed mixer.

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2. *Preparation of biological material*

Gradient centrifugation can be used for the analysis of homogenates and cytoplasmic extracts or of various particulate preparations obtained by conventional fractionation procedures. These are by no means the only applications of the method which promises to become a standard technique of ultracentrifugal analysis for all materials requiring specific chemical or biological assays. Pioneering investigations of this kind are those of HOGEBOOM and KUFF (1954) on a number of macromolecules, of KUFF *et al.* (1955) on crude urease preparations and of LEVINTOW *et al.* (1955) on the systems catalysing glutamine synthesis and glutamyl transfer.

In most early work, the material was layered above the gradient. However, as shown by ANDERSON (1955a), this technique may be complicated by artifacts involving bulk sedimentation of material. In addition, the resulting distribution curves are difficult to analyse correctly unless very thin layers are used or the material resolves into sufficiently well separated bands.

These difficulties do not occur when the material is homogeneously distributed within the whole tube, as has been done by KUFF *et al.* (1956). This is easily done with one of the gradient-producing machines described above, by including the biological material at exactly the same concentration in the two solutions used to prepare the gradients.

The layering technique may, however, be of special interest when complete separation is aimed at. From a theoretical point of view, it may be preferable to layer the material below rather than above the gradient, in order to avoid bulk sedimentation by side-wall impaction.

However, in this case, the radial movement of the particles will cause them to move away from the walls, a phenomenon which may also cause artifacts.

3. *Choice of solutions and physical constants*

Except for the Danish workers who have used a heavy iodinated compound (Diidon) in addition to sucrose (HOLTER *et al.*, 1953; OTTESEN and WEBER, 1955), most authors so far have worked with pure sucrose gradients. These are by no means ideal, especially in isopycnic sedimentation which requires highly hypertonic solutions and it seems desirable therefore to look for other more suitable compounds.

Data such as those of Table 2 (see p. 338) may be useful in guiding a search of this kind. They show, for instance, that a 25 per cent (w/w) solution of a heptahexoside with the same specific gravity as sucrose, would be at the same time isotonic and almost isopycnic with rat-liver mitochondria. In addition to conforming to given requirements of density and tonicity, the solutes must answer a number of other criteria: they should be sufficiently soluble in water, increase the viscosity of the medium to only a moderate extent, exert little effect on *p*H or ionic strength and be largely devoid of denaturing or surface-active properties; they should not inhibit the enzyme systems studied nor interfere with the analytical procedures used.

So far no substance conforming to this description has been found. Some measure of success has been attained in this laboratory with suspensions of colloidal thorium oxide (Thorotrast) brought to isotonicity with sucrose. It has indeed been found that mitochondria equilibrate with a density of approximately 1·10 in this medium; in addition a fairly efficient separation of the denser lysosomes has been achieved. Unfortunately, the Thorotrast particles themselves sediment at an appreciable rate; they are very polydisperse and their sedimentation constant is strongly concentration-dependent. For these reasons, the gradients flatten out rapidly during centrifugation and the results are difficult to analyse quantitatively. In a recent paper, THOMSON and KLIPFEL (1958) have described the use of stabilizing gradients made with polyvinylpyrrolidone dissolved in 0·5 M sucrose.

Another means whereby the osmotic pressure of isopycnic sucrose solutions can be reduced is to replace the water by deuterium oxide. In practice, the advantage is not as great as could be predicted because the particles themselves become slightly denser owing to replacement of their water of hydration by heavy water. However, this may become an advantage since particles having the same density in water solutions, may become separable in heavy water solutions, if they differ in their degree of hydration. Such has indeed been found to be the case for mitochondria and lysosomes.

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Pending improvements of the type indicated above, sucrose will probably continue to be used by many workers. To assist in the preparation of suitable media, the densities and viscosities of sucrose solutions have been listed in Table 3.

TABLE 3

Density and Viscosity of Aqueous Sucrose Solutions. Values Calculated by Interpolation from Data Given by LANDOLT-BORNSTEIN (1923) *and* BATES (1944)

Density		*Concentration*			*Viscosity*		
0°	20°	% w/w	molarity at 20°	molality	0°	5°	20°
1·000					1·79	1·52	1·005
1·010	1·008	2·49	0·073	0·075	1·90	1·60	1·06
1·020	1·017	4·92	0·146	0·151	2·04	1·72	1·13
1·030	1·027	7·32	0·220	0·231	2·21	1·86	1·22
1·040	1·037	9·68	0·293	0·313	2·41	2·02	1·31
1·050	1·047	12·01	0·367	0·399	2·64	2·22	1·43
1·060	1·056	14·31	0·441	0·488	2·91	2·44	1·55
1·070	1·066	16·57	0·516	0·580	3·23	2·70	1·70
1·080	1·076	18·80	0·591	0·676	3·57	2·97	1·85
1·090	1·085	20·99	0·666	0·776	3·97	3·29	2·03
1·100	1·095	23·15	0·741	0·880	4·45	3·67	2·24
1·110	1·105	25·28	0·816	0·988	5·00	4·11	2·48
1·120	1·115	27·37	0·891	1·101	5·67	4·63	2·76
1·130	1·124	29·44	0·967	1·219	6·45	5·25	3·09
1·140	1·134	31·47	1·043	1·342	7·38	5·98	3·47
1·150	1·144	33·48	1·119	1·470	8·50	6·85	3·92
1·160	1·154	35·45	1·195	1·605	9·88	7·91	4·45
1·170	1·163	37·40	1·271	1·746	11·6	9·20	5·09
1·180	1·173	39·33	1·348	1·894	13·7	10·8	5·86
1·190	1·183	41·23	1·425	2·050	16·4	12·8	6·80
1·200	1·193	43·11	1·502	2·214	19·8	15·3	7·94
1·210	1·202	44·97	1·580	2·387	24·2	18·5	9·36
1·220	1·212	46·80	1·657	2·570	29·9	22·6	11·1
1·230	1·222	48·61	1·735	2·763	37·3	27·9	13·3
1·240	1·232	50·40	1·814	2·969	47·2	34·9	16·1
1·250	1·242	52·17	1·892	3·187	60·6	44·2	19·7
1·260	1·251	53·92	1·971	3·419	78·9	56·8	24·4
1·270	1·261	55·65	2·051	3·666	105	74·0	30·6
1·280	1·271	57·37	2·130	3·931	141	98·3	39·0
1·290	1·281	59·06	2·210	4·214	195	133	50·3
1·300	1·291	60·74	2·290	4·519	275	184	66·1
1·310	1·301	62·39	2·371	4·847	400	261	88·5
1·320	1·310	64·04	2·452	5·202	597	380	121
1·330	1·320	65·66	2·533	5·586	918	570	169
1·340	1·330	67·27	2·614	6·004	1470	880	242
1·350	1·340	68·86	2·696	6·461	2440	1410	357

4. *Centrifugation*

Both HOGEBOOM and KUFF (1954) and ANDERSON (1955a) have insisted on the necessity of guarding against mechanical or convective disturbances in gradient centrifugation. Artifacts of this kind belong to three groups:

4.1. *Swirling.* Changes in angular velocity generate rotational movements of the fluid in a horizontal plane. This effect causes the fluid to swirl around the tube axis if the latter is vertical and along this axis, if it is horizontal. While the tube swings out or in, both movements occur, together with a third type of swirling in the vertical plane containing the tube axis. If the tube contains a density gradient, any swirling in an axial plane will be opposed by the axially directed gravitational forces which tend to keep each layer in its appointed place. On the other hand, the gradient affords no protection against swirling around the tube axis.

It follows from these considerations that the most harmful effects of acceleration and deceleration are those occurring at very low speed when both the stabilizing forces are minimal and an unopposed component of swirling is generated. Therefore, below a few hundred revolutions per minute acceleration and deceleration of the centrifuge should be very slow, probably no greater than 10 rev/min per min.

HOGEBOOM and KUFF (1954) have described a device by which the Spinco analytical centrifuge can be adjusted to very slow accelerations. A similar device has been used in our laboratory, with the preparative Model L: a 500 Ω (10 W) resistor, a 5000 Ω potentiometer (100 W) and a 100 mA milliammeter are connected in series between the thyratrons and the saturable reactor controlling the current flowing through the drive motor. Adjustment of the current flowing through the d.c. coil of the reactor from 90 to 10 mA with the 5000 Ω potentiometer allows a gradual transition from rapid acceleration to rapid deceleration.

The acceleration from 0 to 500 rev/min should be as slow as possible to avoid artifacts, but as a measure of safety, it seems also advisable to accelerate slowly from 500 to 5000 rev/min. In gradient sedimentation of heavy particles (mitochondria for instance) a large part of the centrifugal force is then applied during acceleration and deceleration and the integral $W = \int_o^t \omega^2 \, dt$ is much greater than the product (time at top speed) $\times$ (ω^2 at top speed). We found it convenient to build a simple speed recorder adapted to the Spinco Model L centrifuge (Fig. 15). A recording milliammeter is connected to the speed control generator of the centrifuge through a cathode follower. It is necessary to change one resistor of the original wiring to ensure that the zero

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of the recorder is independent of the setting of the speed control potentiometer, but this does not influence the operation of the centrifuge.

It should be pointed out that at speeds of a few revolutions per minute the movement of the rotor of the Spinco Model L centrifuge tends to become irregular if a very slow acceleration is wanted, because the inertia of the system is too small in comparison with the variation of torque with the position of the drive motor. Further improvements would require a five to tenfold increase of the inertia of the rotor.

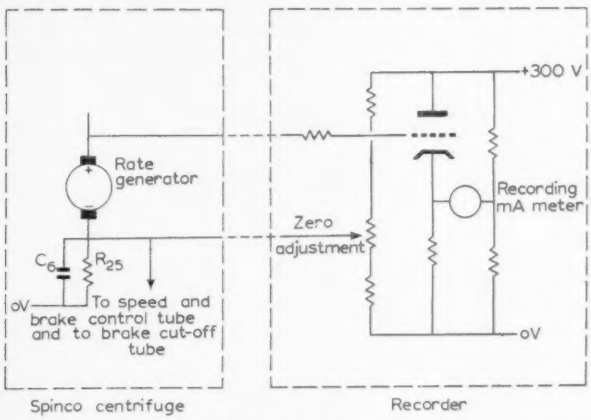

Fig. 15. Speed recorder for Spinco Model L Centrifuge. The resistor R25 (10 kΩ) of the original wiring is replaced by a 500 kΩ resistor. Note that the d.c. power supply of the recorder is independent of the d.c. voltage of the centrifuge.

4.2. *Vibrations.* With the Spinco Model L centrifuge, very little if any high frequency vibrations of the rotor occur. However, at low speeds, a wobbling movement sets in which may acquire a considerable amplitude. This arises from the fact that the elastic axis on which the rotor rests is never quite straight and consequently the equilibrium positions of the rotor at rest and while spinning at high speed are not identical. The transition occurs below 2000 or 3000 rev/min. We suspected that this movement could explain some artifacts which were observed when the centrifuge had been accelerated and decelerated very slowly so that it had remained a long time in the transition state. A stabilizer (Figs. 16 and 17) was therefore built to prevent wobbling of the rotor at low speeds.

A hard aluminium cone with a steel tip is screwed on the top of the SW 39 rotor. When the stabilizer is in operation, the cone of the rotor is adapted inside a hollow steel cone of slightly wider angle, with a

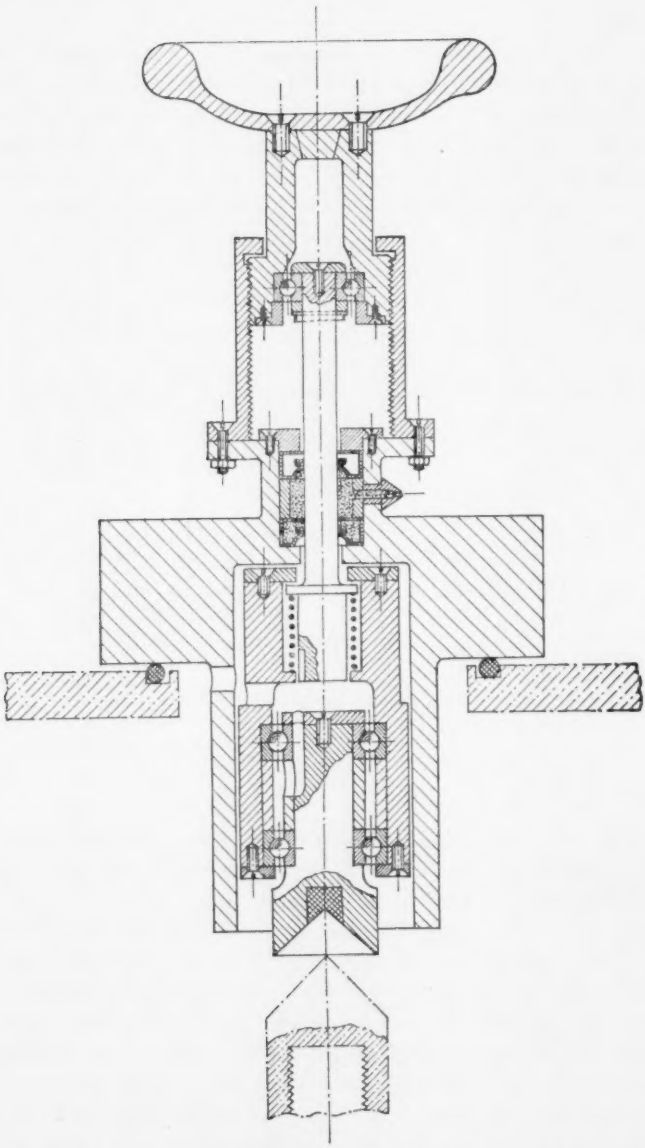

Fig. 16. Diagram of stabilizer. For description, see text.

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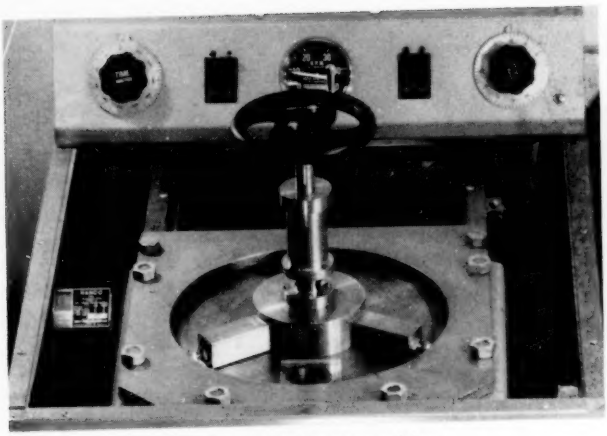

Fig. 17. Stabilizer in position.

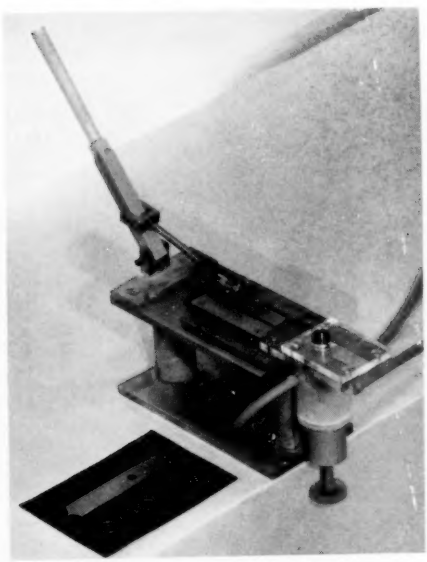

Fig. 18. Tube sectioning device.

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piece of bronze forming the apex. This cone rotates on ball-bearings contained in a steel cylinder, which can be moved up and down by the screw and the handle at the top of the stabilizer. The cylinder as well as the cavity in which it slides are carefully machined to insure an easy movement without appreciable lateral play. To avoid excessive pressure on the rotor, the cylinder hangs from the screw system and slides down by its own weight (about 1·5 kg); slightly more pressure (up to 3 kg) can be transmitted by the screw through the helical spring. To make an air-tight connexion between the screw and the moving cylinder a vacuum seal is placed around the connecting rod. In the lower part of the stabilizer, holes have been drilled in the fixed part and in the moving cylinder to allow a rapid evacuation of the air when the stabilizer is used under vacuum.

The lid of the centrifuge chamber is replaced by a steel plate fitted with two openings: a small inspection port-hole closed with a Perspex plate and an "O" ring, and an 8·0 cm hole closed by the stabilizer resting on an "O" ring. The stabilizer is centred by three arms of variable length.

The stabilizer is lowered before the centrifuge is started; it is raised rapidly when the centrifuge reaches 3000–4000 rev/min; after a few periods of slow and smooth nutation the rotor assumes its stable position. To stop the centrifuge, it is first decelerated to 3000 rev/min; the stabilizer is rapidly lowered on the centrifuge which can then be decelerated to rest slowly and without wobbling.

4.3. *Thermal convection.* Temperature changes are probably a major source of artifact in ordinary centrifugation, the slight differences in density due to the temperature gradient being considerably magnified by the centrifugal field. In gradient centrifugation, convection is effectively prevented by the density gradient. With the Spinco preparative centrifuge, no troubles which could be ascribed to thermal effects have been observed during short centrifugations. Some heating occurs after one or two hours at top speed but it is not sufficient to disturb the steep concentration gradients (0·01 per cm) of viscous fluid used in isopycnic sedimentation.

5. *Sampling*

Several authors have used simple aspiration to remove successive samples from the centrifuge tubes. Disturbance of the lower layers is kept minimal if the tip of the pipette or needle is bent at 90° or inverted. Other authors rely on more elaborate devices which are of two varieties. In those described by Hogeboom and Kuff (1954), Anderson (1955a) and Phelpstead and Roodyn (1957), a solution of high density is injected into the bottom of the tube, thus lifting the entire tube contents. An appropriate collector is fitted on the top of the tube, making it

possible to separate each fraction as it comes up. Other workers use a cutting device of the type described by RANDOLPH and RYAN (1950). Fig. 18 shows a modification of this instrument developed in this laboratory.* The tube is kept cool during fractionation by a jacket through which iced water is circulated. The knife tip is lance-shaped so as to minimize the flattening of the Lucite tube during cutting.

6. *Mathematical procedures*

The procedure adopted by other authors in computing the function $u(x)$ (see p. 348) is to express the density and viscosity of the medium as suitable functions of x and to introduce these functions in the velocity equation. From this, one may calculate values of Ψ and, after integration, of Φ (see equations 42 and 43, p. 348). The resulting equations are, however, fairly complex (see for instance KUFF *et al.*, 1956; THOMSON and MIKUTA, 1954) and the calculations extremely tedious and laborious. In our experience such computations may be simplified to a considerable extent by eliminating all steps irrelevant to the main purposes and by resorting as much as is feasible to purely numerical methods. In addition, it has been found convenient to use the density, as the independent variable whenever possible. For this reason, a special table, which contains all the necessary information concerning sucrose solutions, has been prepared by interpolating from the best available data (Table 3, p. 357).

6.1. *Gradient differential centrifugation.* If the gradient does not include the value ρ_e, as is generally the case in gradient differential centrifugation, $u(x)$ is always positive within the range of x considered and $B/u(x)$ is continuous. If in addition the gradient is linear, numerical methods are applicable throughout. These are illustrated by the example of Tables 4 and 5, which give the main steps in the calculation for a linear gradient of sucrose at 0° from 0·25 to 0·88 M over a distance of 11·5 to 20·0 cm. This is similar to the gradient used by THOMSON and co-workers (1954, 1956, 1957), which is illustrated in Figs. 8 and 9 (see p. 350). The latter were, however, constructed by means of the experimental values for the density and viscosity of the medium given by THOMSON and co-workers.

By interpolation in Table 3, it is found that the gradient extends from $\rho_m = 1{\cdot}0341$ to $\rho_m = 1{\cdot}1185$, and has an inverse slope of 1·007 cm per 0·01 density unit. The values of x corresponding to the round values of density are easily obtained. These data are tabulated in Table 4, with the viscosity obtained directly from Table 3, and the values of $B/u(x) = \eta/(\rho_e - \rho_m)x$ are computed (column 4).

The first and second differences of $B/u(x)$ are calculated. The value

* The general design of this instrument was modelled on a cutter in use in Dr. BLASCHKO's laboratory at Oxford.

TABLE 4

$10^2\rho_m$	x	$10^2\eta$	$\frac{10^2\eta}{(1{\cdot}22-\rho_m)x}$	Δ^1	Δ^2	$\int_{101}^{10^2\rho_m}$	Δ^1	Δ^2
101	9·07	1·90	0·998		(20)			
				14				
102	10·08	2·04	1·012		23	1·005		
				37			1·029	
103	11·09	2·21	1·049		21	2·034		0·047
103·41	11·50		1·070	58		2·469	1·076	
104	12·09	2·41	1·107		20	3·110		
				78				
105	13·10	2·64	1·185		27	4·254		
				104				
106	14·11	2·91	1·289		31	5·489		
				135				
107	15·12	3·23	1·424		23	6·843		
				158				
108	16·12	3·57	1·582		43	8·343		
				201				
109	17·13	3·97	1·783		60	10·021		
				261				
110	18·14	4·45	2·044		70	11·929		
				331				
111	19·14	5·00	2·375		108	14·132		
111·85	20·00			439				
112	20·15	5·67	2·814		134	16·716		
				573				
113	21·16	6·45	3·387					

$B/u(11{\cdot}5)$ at the meniscus is obtained by interpolation with the aid of the well known Stirling formula:

$$y = y_o + h\,\frac{\Delta^1_{-1/2} + \Delta^1_{1/2}}{2} + \frac{h^2}{2}\,\Delta^2_0 \qquad \ldots.(49)$$

where y is the unknown interpolated result,

y_o, the given datum nearest to the unknown result (1·049 in the example),

h, the difference of the independent variable ($10^2\Delta\rho$ or 0·41 in this case),

$\Delta^1_{1/2}$ and $\Delta^1_{-1/2}$, the first differences immediately above and below y_o,

Δ^2_0, the second difference at y_o.

In this case,

$$y = 1{\cdot}049 + 0{\cdot}41 \times 0{\cdot}0475 + \frac{0{\cdot}41^2}{2} \times 0{\cdot}021 = 1{\cdot}070$$

The function $\Psi(11{\cdot}5)/\Psi(x)$ can be computed immediately from the ratios of the numbers of column 4 to the value for $x = 11{\cdot}5$. The results for $\gamma = 0$, 0·5 and 1 are given in Table 5.

TABLE 5

x	$\frac{u(11{\cdot}5)}{u(x)}$	$\left(\frac{11{\cdot}5}{x}\right)^{\frac{1}{2}} \frac{u(11{\cdot}5)}{u(x)}$	$\frac{11{\cdot}5u(11{\cdot}5)}{xu(x)}$	$\Phi(x) - \Phi(11{\cdot}5)$
11·50	1·000	1·000	1·000	0
12·09	1·035	1·009	0·983	0·053
13·10	1·107	1·037	0·971	0·147
14·11	1·205	1·088	0·982	0·248
15·12	1·331	1·161	1·013	0·359
16·12	1·479	1·250	1·055	0·482
17·13	1·666	1·366	1·118	0·620
18·14	1·910	1·522	1·211	0·777
19·14	2·220	1·720	1·332	0·958
20·15	2·630	1·987	1·501	1·170

The function $\Phi(x)$ is obtained by numerical integration starting from $\rho_m = 1{\cdot}010$. The formula used is the integral of the Bessel interpolation formula:

$$\int_{z_0}^{z_0+1} y(z)\,\mathrm{d}z = y_o + \tfrac{1}{2}\Delta^1_{1/2} - \tfrac{1}{24}(\Delta^2_0 + \Delta^2_1) \qquad \ldots.(50)$$

With the values of Table 4 one obtains for instance:

$$\int_{107}^{108} f(\rho)\,\mathrm{d}\rho = 1{\cdot}424 + \frac{0{\cdot}158}{2} - \frac{0{\cdot}023 + 0{\cdot}043}{24} = 1{\cdot}500$$

The cumulated results are given in column 7. The first and second differences near 11·5 cm are used to interpolate (by formula 49) the value of the integral at 11·5 cm, which is 2·469. Subtraction of this number from the values of column 7 gives the integral $\int_{103{\cdot}41}^{10^2\rho} f(\rho)\,\mathrm{d}\rho$. To obtain the integral $\Phi(x) - \Phi(x_o)$, the differences are multiplied by $1/B$ and by the gradient factor (cm per 0·01 density unit). In this case $B = (2{\cdot}28 \times 10^{-2})/(1{\cdot}22 - 1{\cdot}0341) = 0{\cdot}1225$ and the gradient factor is 1·007. The values of $\Phi(x) - \Phi(11{\cdot}5)$ are given in Table 5.

It is seen that this method of computation is the same as the procedure suggested by equation (41), except that the coefficients of the polynomial are not explicitly evaluated. Both methods fail, however, when $u(x)$ takes a null value in the range of x considered, which is the case in isopycnic experiments. $1/u(x)$ is discontinuous and can no longer be represented by a polynomial, and purely numerical methods

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become very inaccurate near ρ_e unless a large number of closely spaced values of $1/u(x)$ are computed.

6.2. *Isopycnic gradient centrifugation.* The simplest procedure in the case of isopycnic sedimentation is illustrated by Tables 6 and 7, which refer to the gradient described above and illustrated by Figs. 10 and 11 (see p. 351). A table (Table 6) is prepared with the first three columns

TABLE 6

ρ_m	x	$\eta 10^2$	$\frac{\eta}{x}10^2$	Δ^1	Δ^2	$\frac{\eta}{x}10^2$ calc.
1·17	4·14	11·6	2·80			3·37
				0·03		
1·18	4·84	13·7	2·83		0·11	3·16
1·1895	5·50			0·14		
1·19	5·53	16·4	2·97		0·07	3·11
				0·21		
1·20	6·23	19·8	3·18		0·10	3·22
				0·31		
1·21	6·93	24·2	3·49		0·12	3·49
1·216	7·35			0·43		
1·22	7·63	29·9	3·92		0·13	3·92
				0·56		
1·23	8·32	37·3	4·48		0·19	4·49
				0·75		
1·24	9·02	47·2	5·23		0·25	5·26
1·2483	9·60			1·00		
1·25	9·72	60·6	6·23		0·34	6·17
				1·34		
1·26	10·42	78·9	7·57			

similar to Table 4. In the fourth column the values of η/x are tabulated and approximated by a polynomial. This is easily obtained from the first and second differences by:

$$y = y_o + \frac{\Delta^1_{1/2}}{\Delta\xi}(\xi - \xi_0) + \frac{\Delta^2_1}{2(\Delta\xi)^2}(\xi - \xi_0)(\xi - \xi_1) \quad \ldots.(51)$$

where $\Delta\xi$ is the constant interval between successive values of ξ. In this case $1/\Delta\xi = 1{\cdot}434$ and, starting from $\xi_0 = 6{\cdot}93$,

$$\frac{\eta}{x}10^2 = 3{\cdot}49 + 1{\cdot}434 \times 0{\cdot}43(x - 6{\cdot}93) + \\ + (1{\cdot}434)^2 \times 0{\cdot}08(x - 6{\cdot}93)(x - 7{\cdot}63)$$

As Δ^2 is not quite constant, the average value 0·16 is taken over the range 6·23–9·02. The development of the above expression gives

$$\frac{\eta}{x}10^2 = 1{\cdot}434(5{\cdot}520 - 1{\cdot}240x + 0{\cdot}1147x^2)$$

The values computed in that way are listed in the last column of Table 6. It is seen that the above equation gives a satisfactory fit within the range of x from 5·5 to 9·6 cm.

The equilibrium distance, where $\rho_m = 1{\cdot}216$, is 7·35 cm. Therefore

$$(\rho_e - \rho_m)10^2 = -1{\cdot}434(x - 7{\cdot}35)$$

and

$$\frac{B}{u(x)} = \frac{\eta}{(\rho_e - \rho_m)x} = \frac{-5{\cdot}520 + 1{\cdot}240x - 0{\cdot}1147x^2}{x - 7{\cdot}35} = \frac{m + nx + px^2}{x + q}$$

The integral of this function is

$$B\Phi(x) = \int \frac{B}{u(x)}\,dx = (m - nq + pq^2)\ln(x + q) + (n - 2pq)(x + q) + \frac{p}{2}(x + q)^2$$

$$= -0{\cdot}5992 \log_{10}[\pm(x - 7{\cdot}35)] - 0{\cdot}446(x - 7{\cdot}35) - 0{\cdot}005735(x - 7{\cdot}35)^2$$

In equilibrium sedimentation, it should be assumed that $\gamma = 0$. Therefore $\Psi(x) = u(x)$. The values of $u(x)/B$ are given in Table 7, as

TABLE 7

x	$\frac{u(x)}{B}$	$\frac{\Psi(5{\cdot}5)}{\Psi(x)}$	$B\Phi(x)$	$\Phi(x) - \Phi(5{\cdot}5)$
5·50	0·853	1·00	−0·972	0
6·00	0·611	1·40	−0·284	5·9
6·50	0·369	2·31	0·761	14·8
6·80	0·230	3·71	1·784	23·7
7·00	0·142	6·01	2·881	33·1
7·20	0·0591	14·4	5·003	51·3
7·30	0·0194	44·0	7·818	75·5
7·35	0	∞	∞	∞
		$\frac{\Psi(9{\cdot}6)}{\Psi(x)}$		$\Phi(x) - \Phi(9{\cdot}6)$
7·35	0	∞	∞	∞
7·40	−0·0190	28·3	7·774	96·0
7·50	−0·0561	9·57	4·869	71·1
7·70	−0·126	4·26	2·569	51·3
8·00	−0·221	2·43	0·807	36·2
8·50	−0·352	1·53	−0·953	21·1
9·00	−0·463	1·16	−2·195	10·4
9·50	−0·525	1·02	−3·216	1·6
9·60	−0·537	1·00	−3·404	0

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well as the values of $B\Phi(x)$ calculated by the above integral. The two parts of the tube above and below ρ_e are of course considered separately for the computation of the definite integrals $\Psi(x_i)/\Psi(x)$ and $\Phi(x) - \Phi(x_i)$.

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